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THE

BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:00 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 9:00 A.M. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. Any person interested in the Laboratory may be elected by the Trustees to a group to be known as Associates of the Marine Biological Laboratory.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE TREASURER

The following tables reflect the financial position of the Laboratory.

The first table summarizes the position of the securities in the Endowment Funds. The market value of January 1, 1954, was $1,153,995.88 in comparison to the market value shown on January 1, 1953, of $1,166,063.74. The average yield on the securities was 4.77% on book values and 4% on market values, or slightly higher than was the case in 1952.

The second table gives the Balance Sheet of the Laboratory as of December 31, 1953. It reflects the entries which were necessary in connection with the taking-by-condemnation proceedings by the U. S. Navy of the Bar Neck property. While the final settlement of this matter is still subject to negotiation or litigation, $75,000 was deposited in the courts and paid over to the Laboratory. This resulted in our reducing the land and buildings by the amount at which this property had been carried and "other investment securities" were increased by about $75,000 due to the investment of these funds. As the matter has not been finally settled an item is carried on the liability side of $55,247.08 pending final determination; this item representing the difference between the original book value of the property and the amount paid to the Laboratory by the Government, and will be further adjusted later to reflect the actual total amount finally received from the Government for the property and to cover expenses incurred by the Laboratory in the condemnation proceedings.

The third table is a condensation of the auditor's income and expense statements for the past five years. As I noted last year, this table points out strongly the need of the Laboratory for additional income and the importance of continued efforts by everyone connected with the Laboratory to secure special donations each year. Your officers and the Executive Committee are giving this matter particular attention and will undoubtedly report to you from time to time. It is obvious that without these special donations, it would have been impossible for the Laboratory to continue to make available the facilities and services necessary for biological research and instructions.

The other tables submitted call for no comment.

ENDOWMENT FUNDS

January 1, 1954

	Book Value	%	Market Value	%
Bonds				
U. S. Government	$268,772.56	27.75	$ 260,944.00	22.62
Railroad	54,613.02	5.63	57,560.00	4.98
Public Utility	90,488.75	9.34	86,350.00	7.48
Industrial	139,128.13	14.37	135,100.00	11.71
Total Bonds	553,002.46	57.09	539,954.00	46.79
Preferred Stocks	103,805.13	10.72	96,816.00	8.39
Common Stocks	310,953.08	32.11	516,390.00	44.75
Total Securities	967,760.67		1,153,160.00	
Principal Cash	835.88	.08	835.88	.07
TOTALS	$968.596.55	100.	$1,153,995.88	100.

The average yield on the securities was 4.77% on book value, 4.00% on market value.

Marine Biological Laboratory Balance Sheet, December 31, 1953

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of the Hanover Bank, New York, Trustees		$ 968,596.55	
Securities and Cash in Minor Funds		21,701.36	$ 990,297.91
Plant Assets:			
Land		$ 97,030.04	
Buildings		1,490,726.83	
Equipment		338,588.94	
Library		446,071.11	
		$2,372,416.92	
Less Reserve for Depreciation		885,781.84	$1,486,635.08
Current Assets:			
Cash		$ 23,352.94	
Mortgage Note Receivable		2,200.00	
Accounts Receivable		42,341.67	
Inventories:			
Supply Department	$ 52,565.88		
Biological Bulletin	17,014.61	69,580.49	
Investments:			
Devil's Lane Property	37,510.74		
Stock in Gen. Biol. Supply House, Inc.	12,700.00		
Other Investment Securities	122,149.39		
Retirement Fund	24,017.72	196,377.85	
Prepaid Insurance		6,808.33	
Items in Suspense (Debits)		3,160.86	343,822.14
			$2,820,755.13

Liabilities

Endowment Funds:			
Endowment Funds	$ 965,729.75		
Reserve for Amortization	2,866.80	$ 968,596.55	
		21,701.36	$ 990,297.91
Plant Funds:			
Mortgage Note Payable		5,000.00	
Donations and Gifts	1,409,441.81		
Other Investments in Plant from Gifts and Current Funds	72,193.27	1,481,635.08	1,486,635.08
Current Liabilities and Surplus:			
Accounts Payable		36,593.15	
Items in Suspense (Credits)		2,939.37	
Bar Neck Settlement Account		55,247.08	
Current Surplus		249,042.54	343,822.14
			$2,820,755.13

MARINE BIOLOGICAL LABORATORY

Five Year Condensed Income and Expense Statement

Reference—Exhibit B and B-1 Auditors' Reports—1949 through 1953

	1953	1952	1951	1950	1949	Total
Income from Endowments	$ 46,261.43	$ 44,115.00	$ 44,898.43	$ 46,431.59	$ 40,347.15	$ 222,053.85
Income from Investments, Including Retirement Fund and Mortgage Rec.	23,195.75	21,635.50	23,184.86	22,844.19	22,593.55	113,453.85
Income from Fees and Dues, including Instruction, Research, Library	56,852.85	46,695.76	46,633.00	47,888.61	44,921.33	242,991.55
Income from Mess, Dormitories, Supply, Biol. Bulletin, Sundry—Net	28,289.49	19,690.73	12,943.63	21,601.35	27,499.18	110,024.38
Income from Rents, including Bar Neck	. . . . .	7,333.26	8,047.42	7,090.89	6,330.00	28,801.57
TOTAL ORDINARY INCOME . . . A	154,599.52	139,470.25	135,707.34	145,856.63	141,691.21	717,324.95
Income by Special Donations	49,049.00	65,242.69	77,730.00	43,130.66	37,459.45	272,611.80
TOTAL INCOME . . . X	$203,648.52	$204,712.94	$213,437.34	$188,987.29	$179,150.66	$ 989,936.75
Buildings, Grounds, and Other Property Expense	$ 55,159.57	$ 51,020.70	$ 56,353.84	$ 53,066.36	$ 41,591.16	$ 257,191.63
Research Service Expense—Apparatus and Chemical Rooms	33,613.24	24,312.98	25,492.99	25,244.54	23,901.70	132,565.45
Instruction, Research and Library Expense	45,968.79	34,632.76	34,083.50	34,077.76	30,587.06	179,349.87
General Expenses, Administration, Pension Fund, Bank Charges, etc.	42,790.76	39,396.93	41,375.04	37,623.08	43,650.91	204,836.72
	177,532.36	149,363.37	157,305.37	150,011.74	139,730.83	773,943.67
Depreciation	35,935.10	34,359.93	32,181.00	29,160.29	27,919.94	159,556.26
	213,467.46	183,723.30	189,486.37	179,172.03	167,650.77	933.499.93
Plant Additions from Current Funds	20,630.33	19,360.99	16,767.59	17,775.06	33,408.40	107,942.37
TOTAL ORDINARY EXPENSE . . . B	234,097.79	203,084.29	206,253.96	196,947.09	201,059.17	$1,041,442.30
Special Expenditures from Donations	. . . . .	10,433.11	9,705.88	5,129.49	. . . . . .	25,268.48
	234,097.79	213,517.40	215,959.84	202,076.58	201,059.17	1,066,710.78
Plant Additions from Donations	. . . . .	23,722.13	44,020.64	. . . . .	. . . . .	67,742.77
TOTAL EXPENSE . . . Y	$234,097.79	$237,239.53	$259,980.48	$202,076.58	$201,059.17	$1,134,453.55
TOTAL NET (Y − X)	**30,449.27**	**32,526.59**	**46,543.14**	**13,089.29**	**21,908.51**	**144,516.80**
Delete Depreciation	35,935.10	34,359.93	32,181.00	29,160.29	27,919.94	159,556.26
Total Net, less Depreciation	5,485.83	1,833.34	**14,362.14**	16,071.00	6,011.43	15,039.46
Operating Net (B − A)	**79,498.27**	**63,614.04**	**70,546.62**	**51,090.46**	**59,367.96**	**324,117.35**
Delete Depreciation	35,935.10	34,359.93	32,181.00	29,160.29	27,919.94	159,556.26
Operating Net, Less Depreciation	**$ 43,563.17**	**$ 29,254.11**	**$ 38,365.62**	**$ 21,930.17**	**$ 31,448.02**	**$ 164,561.09**

Marine Biological Laboratory Current Surplus, December 31, 1953

Balance, January 1, 1953			$221,726.86
Add:			
Prior Year Adjustments, Net Recovery		$ 2,293.51	
Transfer from Plant Funds, Net Book Value of Bar Neck Property		19,536.34	
Reserve for Depreciation, 1953, Charged to Plant Funds		35,935.10	57,764.95
			$279,491.81
Deduct:			
Excess of Expense over Income for Year, as		9,818.94	
Payments during Year from Current Funds for Plant Assets:			
Buildings	$ 5,296.64		
Equipment	1,097.49		
Library	14,236.20	20,630.33	30,449.27
Balance, December 31, 1953			$249,042.54

Summary of Bank Balance

Cash Balance, January 1, 1953		
The Hanover Bank	$25,547.59	
Falmouth National Bank	2,547.53	$ 28,095.12
Receipts		563,657.05
		591,752.17
Payments		576,517.30
Cash Balance, December 31, 1953		
Hanover Bank	14,557.93	
Falmouth National Bank	676.94	$ 15,234.87

	Balances Jan. 1, 1953	Received	Paid	Balances Dec. 31, 1953
Allen R. Memhard Fund	$ 86.65	$ 27.50		$ 114.15
Rev. Arsenious Boyer Fund	177.58	148.76	$ 170.00	156.34
Lucretia Crocker Fund	1,192.38	553.16		1,745.54
G. H. A. Clowes Beach Fund	1,000.00			1,000.00
F. R. Lillie Memorial Research Fund	6,393.07	225.00		6,618.07
Retirement Fund	2,897.95			
Bio Club Scholarship Fund	794.75	579.78	1,133.75	240.78
Lalor Foundation Fellowship Fund	976.50	6,000.00	6,117.48	859.02

V. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith the report of the sixty-sixth session of the Marine Biological Laboratory.

The success of the Marine Biological Laboratory over the past sixty-six years in research and teaching and the development of its plant and facilities surely fulfills the fondest hopes of the founding group. Throughout this period the Laboratory, through its mode of operation, has fostered and encouraged the freest development of the research ideas of its investigators who themselves, as members of the Corporation, determine its policies.

There has been no suggestion or thought of changing the traditional policies of the Laboratory. In fact, there are many good reasons for continuing along the lines established at the founding of the Laboratory. Probably the most cogent of these is the great need for emphasis on basic research in all fields of science in this country. Fortunately, most of the foundations, including those in the medical sciences and the various governmental agencies controlling research funds, have very enlightened leadership at the policy-making and fund-granting levels. Thus it has been possible for the Marine Biological Laboratory to secure significant support from such sources under grants or contracts. This support is listed under item 1 in this report.

Also, there have been significant contributions listed below from friends of the Laboratory. Included in this is a contribution from the M.B.L. Associates who number 128.

1. Grants, Contracts and Contributions:

These are assuming great importance in the support of the Laboratory. This past year they totaled $108,488.37.

Grants:	
Rockefeller Foundation, general support	$17,500.00
Rockefeller Foundation, special apparatus	5,000.00
National Science Foundation	10,000.00
American Philosophical Society	1,800.00
American Cancer Society	5,350.00
New York Zoological Society	4,000.00
F. R. Lillie Fellowship	1,500.00
National Institute of Health—Studies of Arbacia egg and sperm cells	1,080.00
Contracts:	
Office of Naval Research, studies on marine biology	15,000.00
Office of Naval Research, investigation of environmental factors influencing certain marine biological populations in the Woods Hole area	8,639.50
Office of Naval Research on encephalization during ontogency in *Ameiurus nebulosus*	2,224.00
Office of Naval Research, research in the fundamental properties and functions of nerve tissue by use of the giant axon of the squid	7,695.87
Office of Naval Research, osmoregulation in marine animals	1,000.00
Atomic Energy Comission, research employing radioisotopes in an investigation of the biochemistry of cell nuclei	13,400.00
Atomic Energy Commission, research on the physiology of marine organisms, using radioisotopes	5,400.00
Contributions:	
Mrs. Margaret Meigs	1,000.00
Eli Lilly and Company	5,000.00
Miscellaneous	1,274.00
MBL Associates	1,625.00

2. Plant Improvements:

The great increase in the amount and variety of electrical equipment use in the Laboratory in recent years has resulted in an overloading of some of the electrical circuits. Over the past two years, added lines have been installed and others have been changed to take care of the additional load. Also, new transformers have been installed on the main incoming power lines which are important in the maintenance of constant voltages. Mr. Robert Kahler, Manager of Maintenance, and his staff merit special commendation for their handling of various emergencies which have occasionally occurred in the past due to overloading of lines. With further modifications and changes, it can be confidently anticipated that power failures and voltage fluctuations will be avoided in the future.

3. Operations Analysis:

During the past year, a thorough-going operations analysis of the Laboratory was carried out by Dr. Richard Parmenter who transmitted his final report to the Executive Committee in February (1954). Some of the recommendations made in this report have already been put into effect. Others have been referred to the various appropriate standing committees and to special committees for their consideration. It is anticipated that as a result of this report and the implementation of many of the recommendations, improvements in the operation of the Laboratory will result with continuing information available on the financial operations.

4. Changes in the Dining Hall:

During the past summer, the Dining Hall operated on a self-service basis in a manner that appeared generally satisfactory to the investigators and students. Definite economies resulted. This balanced on a cash basis the financial operation of the Dining Hall which had showed modest deficits in its two preceding summers.

5. Instruction:

Dr. Francis T. Haxo found it advisable to resign as Head of the course in Marine Botany and will be succeeded by Dr. Harold Bold.

The Laboratory was very fortunate in receiving important support from the New York Zoological Society (Mr. Fairfield Osborn, President) for the course in Marine Ecology for the first three years of its operation. This course has been an outstanding success under the leadership of Dr. Bostwick H. Ketchum. It will devolve upon the officers of the Laboratory to seek support elsewhere for the course after 1954.

6. Bar Neck Property:

During the past year, negotiations have continued with the Navy to achieve a settlement for adequate compensation for the Bar Neck Property. Mr. Swope, President of the Corporation, has devoted a great deal of time, thought and energy to this problem. It is hoped that an equitable agreement can be reached in the near future and thus avoid the necessity of long court proceedings.

Respectfully submitted,
Philip B. Armstrong,
Director

1. Memorial

Edwin Grant Conklin

1863–1952

E. G. Butler

With the death of Professor Edwin Grant Conklin the biological sciences lost an outstanding leader and the Marine Biological Laboratory one of its earliest and most eminent members. He was born in Waldo, Ohio, November 24, 1863. After a brief illness, he died in Princeton, New Jersey, November 21, 1952.

Most of his youth was spent near Delaware, Ohio, where his father was a country doctor. Here he attended the public schools and later the Ohio Wesleyan University, from which he was graduated in 1885. Although Conklin early became interested in the natural sciences, close second interests, beginning with his undergraduate years, were those in literature and philosophy. With money earned by teaching in a one-room district school during an interval in his college course he began his private library of books in the classics, philosophy, religion, history and science. His deep interest in literature remained with him to the end of his days and his lectures and writings were frequently enlivened by quotations of prose and poetry, much of it committed to memory as a young man.

On graduation from college he took a position at Rust University, Holly Springs, Mississippi, a school for the education of negroes operated by the Methodist Church. There for three years he taught a great variety of subjects, including all of the science offered. It was during this period that he decided to make biology his life work and accordingly he entered the Johns Hopkins University in 1888. Until he enrolled at Johns Hopkins Conklin had had no laboratory training in biology, his knowledge of the subject having come from field collecting, museum work and text-book study. Consequently, during his first year at the Johns Hopkins he took practically all of the undergraduate courses in biology, as well as beginning graduate study under Professor W. K. Brooks. His principal field of study at Johns Hopkins was animal morphology and he received the Ph.D. degree in 1891.

Woods Hole early became Conklin's scientific home. He first came here in the summer of 1890, occupied a research table at the United States Fish Commission, and thus began an association with Woods Hole which was to continue throughout the rest of his life. After two summers at the Fish Commission, he transferred in 1892, on the invitation of Professor Whitman, to the Marine Biological Laboratory. Altogether, his association with Woods Hole as an active research worker covered a span of sixty-two years, a record which I believe is unequaled.

It was during his first summer at Woods Hole that he became interested in the development of the gastropods. He found *Crepidula* abundant and favorable for study and began his work on the embryology and cytology of this form, an investigation which, in one way and another, was to occupy him for many years and to result in numerous important contributions. It was his first summer's research on the early development of *Crepidula*, dealing especially with cell lineage, that formed the subject of his doctoral thesis. It is noteworthy that this was a problem of Conklin's own choice and one which did not at first receive the wholehearted approval of his Johns Hopkins preceptor, Professor Brooks. However, around the turn of the century the method of carefully following the fate of individual cells from the first cleavage of the fertilized egg on through later cleavages to the establishment of parts of the embryo became of prime importance in solving problems concerned with the differential localization of organ-forming substances. In such work Conklin, along with E. B. Wilson and F. R. Lillie, became a leader.

Conklin's research over the years dealt with many different organisms and covered a wide range of subjects in cytology and embryology, but he achieved eminence particularly for his work on the gastropods, the ascidians and *Amphioxus*. He often told of the gratification and delight he experienced when, on examining the eggs of a number of Woods Hole ascidians, he found the naturally-pigmented egg of *Cynthia partita*. As he once wrote, this discovery "modified the whole course and purpose" of his work. It enabled him greatly to extend his earlier studies on the mosaic type of development and the distribution and fate of organ-forming materials. He published many papers on the normal and experimental embryology of *Cynthia* and his great monograph on the *Organization and Cell Lineage of the Ascidian Egg*, published in quarto with 207 figures (60 in color), stands as a landmark of his research.

In connection with his interests in cell lineage and the mosaic type of development, he decided to turn his attention to the early embryology of *Amphioxus*. This work was begun during a period of residence at the Zoological Station in Naples and was continued for many years on material which he had collected there. The results were presented in his papers on the development of *Amphioxus*, including both normal and experimental studies, which greatly extended our knowledge of the embryology of this important form. Despite the comprehensive nature of this investigation, Conklin regarded it as incomplete and those who visited him in his laboratory last summer found him hard at work on his *Amphioxus* material.

Early in his research Conklin began to use experimental methods, especially the application of centrifugal force and changes in temperature and pressure, and to study the cytoplasmic alterations which they produced. More than most biologists he was ever conscious of the relation to one another of the descriptive and the experimental methods and the values of each. His deep interest in experimental zoology is exemplified by the fact that he was one of three zoologists, E. B. Wilson and T. H. Morgan being the others, who were initially responsible for the establishment of the *Journal of Experimental Zoology*. He served on the Editorial Board from the first issue in 1904 until his death.

Of deep concern to Conklin throughout his life were the philosophical and social implications of science. His own research, together with extensive reading, beginning in his youth, in philosophy, religion and social problems, led him to much constructive thinking and the formulation of important concepts dealing with embryology and evolution, evolution and religion, teleology, science and ethics and kindred subjects. He was endowed with an extraordinary ability for penetrating thought on philosophical matters and a remarkable talent for clear and forceful exposition. He was a master in bringing in apt quotations from the great works of science and the classics. The titles and subjects of his books reflect his deep concern with fundamental human problems. *Heredity and Environment in the Development of Men* went through six editions and many printings and was translated into Japanese, French and Russian. His later books, the *Direction of Human Evolution, Freedom and Responsibility, What is Man?*, and *Man: Real and Ideal,* received wide acclaim. In all, he published over 250 works on Embryology, Cytology, Evolution and Education. About half of them deal with evolutionary and philosophical matters. He regarded it as an "inestimable privilege . . . to add to the store of knowledge, to seek truth not only for truth's sake but also for humanity's sake, and to have a part in the greatest work of all time . . . the further progress of the human race."

In his teaching career Conklin was associated with four institutions. On completing his graduate studies at Johns Hopkins, he returned to Ohio Wesleyan University where he served as professor for three years. Then followed two years at Northwestern University and twelve years at the University of Pennsylvania. In 1908, on the invitation of Woodrow Wilson, then president of Princeton University, he joined the Princeton faculty as Professor of Biology and Chairman of the Department. Here he remained for the rest of his life, becoming Professor Emeritus in 1933.

One cannot include in a memorial such as this an enumeration of the many honors conferred upon him. He was long a member of the National Academy of Sciences and at one time or another served as president of the leading zoological societies of the country. In 1936 he was president of the American Association for the Advancement of Science. He was elected to membership in a number of foreign academies. Honorary degrees of Sc.D. and LL.D. were awarded him by eight universities. Especially intimate was his relation to the American Philosophical Society, in the activities of which he took a leading part for many years. It is particularly noteworthy that in the long history of this organization, the oldest learned society in America, he was the only person to have been twice elected president.

Conklin was one of the old guard of the Marine Biological Laboratory, of the group intimately associated with the early days of the Laboratory under Whitman and with its development through the years. He became a member of the Corporation in 1894 and was a Trustee from 1897 on, becoming Trustee Emeritus in 1934. His counsel in laboratory affairs was frequently sought. As the records show, he served on many standing and special committees and had much to do in solving the problems and shaping the policies of this institution. Few have had a longer or more intimate connection with the Marine Biological Laboratory than he; no one has been more devoted to its welfare. He was a trustee of the Woods Hole Oceanographic Institution, beginning with its establishment in 1930, and last summer was made an Honorary Trustee. With his deep interest in marine biology, he was a leader in the reorganization of the Bermuda Biological Station in 1925 and served as its president for ten years.

A recital of Conklin's achievements in research, his well-deserved eminence as a scholar, his devoted service to many institutions fails to present the complete picture of Conklin as a man. All who knew him came to feel the spell of his warm personal characteristics, to enjoy his brilliance as a conversationalist, his wit, and especially to treasure the stimulation which came from his liberal philosophical and social outlook. His intellectual horizons were broad; he was ever finding new ideas or new and interesting ways of looking at old ideas.

It was Conklin who was called upon to give the principal address at the dedication of this building in 1925. In the course of this address he said: "Our strongest social instincts are for service; the joy of life is in progress; the desire of all men is for immortality through their work." What better words than these can we find to express our appreciation of his life, his work and his contributions to the Marine Biological Laboratory.

Jeremy Babb

1933–1953

Jeremy Babb was born on January 26, 1933, the son of Mr. and Mrs. Glenn Babb of Bedford, Virginia.

Jeremy had just completed his sophomore year as a premedical student at Princeton University and came directly to Woods Hole to work in the Supply Department of the Laboratory. Here he proved himself an able, willing and conscientious worker. He took an active part in the life around him at the Laboratory; his cheerful and friendly personality made him well liked by all who were associated with him.

It was in the early morning hours of July 9, 1953, as he drove to get squid that he met accidental death.

The members of the Corporation of the Marine Biological Laboratory record with deep sorrow the death of Jeremy Babb and they extend heartfelt sympathy to his family.

2. THE STAFF, 1953

PHILIP B. ARMSTRONG, Director, State University of New York, School of Medicine, Syracuse

SENIOR STAFF OF INVESTIGATION

A. P. MATHEWS, Professor of Biochemistry, *Emeritus,* University of Cincinnati
G. H. PARKER, Professor of Zoology, *Emeritus,* Harvard University

ZOOLOGY

I. CONSULTANTS

F. A. BROWN, JR., Professor of Zoology, Northwestern University
LIBBIE H. HYMAN, American Museum of Natural History
A. C. REDFIELD, Woods Hole Oceanographic Institution

II. INSTRUCTORS

L. H. KLEINHOLZ, Professor of Biology, Reed College, in charge of course
JOHN H. LOCHHEAD, Associate Professor of Zoology, University of Vermont
NORMAN A. MEINKOTH, Associate Professor of Zoology, Swarthmore College
GROVER STEPHENS, Assistant Professor of Zoology, University of Minnesota
JOHN M. ANDERSON, Associate Professor of Zoology, Cornell University
MURIEL SANDEEN, Department of Zoology, Duke University
SEARS CROWELL, Assistant Professor of Zoology, Indiana University
HOWARD T. ODUM, Assistant Professor of Biology, University of Florida

III. LABORATORY ASSISTANTS

WILLIAM E. DOSSEL, Johns Hopkins University
ELIZABETH PAULSEN, Rutgers University

EMBRYOLOGY

I. INSTRUCTORS

S. MERYL ROSE, Associate Professor of Zoology, University of Illinois, in charge of course
WILLIAM E. BERG, Assistant Professor of Zoology, University of California, Berkeley
MAC V. EDDS, JR., Associate Professor of Biology, Brown University
JOHN R. SHAVER, Assistant Professor of Zoology, University of Missouri
J. P. TRINKAUS, Assistant Professor of Zoology, Yale University
EDGAR ZWILLING, Associate Professor of Genetics, University of Connecticut

II. LABORATORY ASSISTANTS

LILLIAN M. YOUNGS, University of North Carolina
SUSUMU ITO, Western Reserve University

PHYSIOLOGY

I. CONSULTANTS

MERKEL H. JACOBS, Professor of Physiology, University of Pennsylvania
OTTO LOEWI, Professor of Pharmacology, New York University, School of Medicine

Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Gyorgyi, Director, Institute for Muscle Research, Woods Hole
E. S. Guzman Barron, Associate Professor of Biochemistry, University of Chicago

II. Instructors

Daniel Mazia, Associate Professor of Zoology, University of California, in charge of course
Stephen Kuffler, Associate Professor of Ophthalmology, Wilmer Institute, Johns Hopkins University Medical School
Max A. Lauffer, Professor and Head of Dept. of Biophysics, University of Pittsburgh
Roger Y. Stanier, Professor of Bacteriology, University of California, Berkeley
H. Burr Steinbach, Professor of Zoology, University of Minnesota
George Wald, Professor of Biology, Harvard University
Andrew Szent-Gyorgyi, Independent Investigator, The Institute for Muscle Research

III. Laboratory Assistant

Paul Bernstein, Department of Zoology, Washington University

BOTANY

I. Consultants

Maxwell S. Doty, Associate Professor of Botany, University of Hawaii
Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Francis T. Haxo, Assistant Professor of Marine Botany, Scripps Institution, University of California, in charge of course
Paul C. Silva, Instructor in Botany, University of Illinois
Richard C. Starr, Instructor in Botany, University of Indiana

III. Laboratory Assistants

Ann Allen, Indiana University
Robert T. Wilce, University of Michigan

IV. Collector

Peter Jennings, Drew University

MARINE ECOLOGY

I. Consultants

W. C. Allee, University of Florida
Alfred C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Bostwick H. Ketchum, Marine Microbiologist, Woods Hole Oceanographic Institution, in charge of course
Edwin T. Moul, Assistant Professor of Botany, Rutgers University
Charles Jenner, Assistant Professor of Zoology, University of North Carolina

III. Assistant

Robert T. Wilce, University of Michigan

THE LABORATORY STAFF, 1953

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
James McInnis, Manager of Supply Department
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Polly L. Crowell
Mrs. Lila Myers
Nancy Shave
Elizabeth Correllus

LIBRARY

Mary A. Rohan
Elizabeth A. Young
Mary Ann Davis
T. E. Tawell
Albert Neal

MAINTENANCE OF BUILDINGS AND GROUNDS

Robert Adams
Arthur Callahan
Robert Gunning
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George A. Kahler
Donald B. Lehy
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James S. Thayer

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
Seaver Harlow
Patricia Philpott

SUPPLY DEPARTMENT

Ruth S. Crowell
Milton B. Gray
A. M. Hilton
Walter E. Kahler
Robert Perry
Patricia M. Conway
Geoffrey Lehy
Robert O. Lehy
Carl Schweidenback
James P. Whitcomb
H. S. Wagstaff

BIOLOGICAL BULLETIN

Donald P. Costello, Managing Editor
University of North Carolina, Dept. of Zoology
Chapel Hill, North Carolina
Catherine Henley, Assistant to the Editor

3. Investigators and Students

Independent Investigators, 1953

Allee, Warder Clyde, Head Professor of Biology, University of Florida
Allen, M. J., Assistant Professor of Zoology, University of New Hampshire
Alscher, Ruth Paula, Associate Professor, Manhattanville College of the Sacred Heart
Amberson, William R., Professor and Head of Department, University of Maryland Medical School
Anderson, John Maxwell, Associate Professor of Zoology, Cornell University
Anderson, Robert S., Professor of Physiology, University of South Dakota
Armstrong, Philip B., Professor of Anatomy, State University of New York, College of Medicine at Syracuse
Ball, Eric G., Professor of Biological Chemistry, Harvard Medical School
Bang, Frederick B., Associate Professor of Medicine, Johns Hopkins University School of Medicine
Barlow, Horace B., Instructor, Wilmer Institute, Johns Hopkins Hospital
Bartlett, James H., Professor of Physics, University of Illinois
Berg, William E., Assistant Professor of Zoology, University of California
Berger, Charles A., Chairman, Dept. of Biology, Fordham University
Bernstein, Maurice H., Jr., Staff Member, Carnegie Institution of Washington
Boettiger, Edward G., Associate Professor of Zoology, University of Connecticut
Borei, Hans G., Professor of Zoology, University of Pennsylvania
Boyarsky, Louis L., Associate Professor of Physiology, University of Kentucky
Brown, Frank A., Jr., Professor and Chairman, Dept. of Biological Sciences, Northwestern University
Brownell, Katherine A., Instructor, Ohio State University
Bruner, Joyce A., Research Associate, State University of Iowa
Butler, Elmer G., Professor of Biology, Princeton University
Cantoni, G. L., Associate Professor in Pharmacology, Western Reserve University
Chaet, Alfred B., Research Assistant, University of Pennsylvania
Chase, Aurin M., Associate Professor of Biology, Princeton University
Cheney, Ralph Holt, Professor of Biology, Brooklyn College
Claff, C. Lloyd, Associate in Surgery, Harvard Medical School
Clark, Arnold M., Associate Professor of Biology, University of Delaware
Clark, Elliot R., Professor Emeritus of Anatomy, University of Pennsylvania
Clowes, G. H. A., Research Director Emeritus, Lilly Research Laboratories
Clement, A. C., Associate Professor of Biology, Emory University
Cohen, Adolph I., Fellow, National Science Foundation, University of California
Cole, Kenneth S., Technical Director, Naval Medical Research Institute
Colwin, Arthur L., Associate Professor of Biology, Queens College
Colwin, Laura, Professor of Biology, Queens College
Cooperstein, Sherwin J., Assistant Professor of Anatomy, Western Reserve University
Cornman, Ivor, Assistant Research Professor in Anatomy, George Washington University
Costello, Donald P., Kenan Professor in Zoology, University of North Carolina
Crowell, Sears, Assistant Professor of Zoology, Indiana University
Csapo, Arpad I., Staff Member and Lecturer, Carnegie Institution of Washington
deVillafranca, George, Instructor in Zoology, Smith College
Dikshit, Padmakar, Nutrition Research Laboratories, Coonoor, India
Dische, Zacharias, Associate Professor of Biochemistry, Columbia University
Edds, Mac V., Jr., Associate Professor of Biology, Brown University
Eichel, Herbert J., Research Associate, Hahnemann Medical College
Fraenkel, Gottfried S., Professor of Entomology, University of Illinois
Gall, Joseph G., Instructor in Zoology, University of Minnesota
Gasteiger, Edgar L., Research Associate, Massachusetts General Hospital
Goldstein, Lester, Research Associate, University of Pennsylvania
Goodall, Marcus C., Independent Investigator, Institute for Muscle Research
Grant, Philip, Research Fellow, Public Health Service, Columbia University

Green, James W., Assistant Professor of Physiology, Rutgers University
Greene, Peter H., Student and University Fellow, University of Chicago
Griffin, Donald R., Professor of Zoology, Cornell University
Grosch, Daniel S., Associate Professor of Genetics, North Carolina State College
Grundfest, Harry, Associate Professor in Neurology, College of Physicians and Surgeons
Guttman, Rita, Assistant Professor of Biology, Brooklyn College
Guyselman, J. Bruce, Instructor in Zoology, Carleton College
Hajdu, Stephen, Independent Investigator, The Institute for Muscle Research
Hamer, Douglas, Biochemist and University Research Fellow, Cancer Research Laboratories, Birmingham, England
Hammond, Warner S., Associate Professor of Anatomy, State University of New York, College of Medicine at Syracuse
Hartman, F. A., Instructor and Professor, Ohio State University
Harvey, Ethel Browne, Independent Investigator in Biology, Princeton University
Harvey, E. Newton, Professor of Physiology, Princeton University
Hayashi, Teru, Associate Professor of Zoology, Columbia University
Haxo, F. T., Assistant Professor of Marine Botany, Scripps Institution of Oceanography
Heilbrunn, L. V., Professor of Zoology, University of Pennsylvania
Henley, Catherine, Research Associate, University of North Carolina
Hickson, Anna Keltch, Research Chemist, Lilly Research Laboratories
Hild, Walther, Fellow Rockefeller Foundation, University of Colorado
Hunter, F. R., Professor and Head of Dept. of Physiology, Florida State University
Huxley, A. F., Assistant Director of Research, Physiological Laboratory, Cambridge, England
Hyman, Chester, Associate Professor of Physiology, University of Southern California
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania
Jenkins, George B., Professor Emeritus of Anatomy, George Washington University
Jenner, Charles E., Assistant Professor of Zoology, University of North Carolina
Jones, E. Ruffin, Professor of Biology, University of Florida
Kao, Chien-yuan, Assistant in Pathology, Cornell University Medical College
Kalckar, Herman M., Dept. Head, Institute for Cytophysiology, University of Copenhagen, Denmark
Kempton, Rudolf T., Chairman, Department of Zoology, Vassar College
Kind, Charles Albert, Assistant Professor of Chemistry, University of Connecticut
Kleinholz, L. H., Professor of Biology, Reed College
Klotz, Irving M., Professor of Chemistry and Biology, Northwestern University
Krahl, Maurice E., Associate Professor of Biological Chemistry, Washington University Medical School
Kuffler, Stephen W., Associate Professor, Wilmer Institute, Johns Hopkins Hospital
Kuntz, Eloise, Assistant Professor of Physiology, Vassar College
Lansing, Albert I., Associate Professor of Anatomy, Washington University Medical School
Lauffer, Max A., Professor and Head of Dept. of Biophysics, University of Pittsburgh
Lazarow, Arnold, Associate Professor, Western Reserve University
Levy, Milton, Associate Professor of Chemistry, New York University College of Medicine
Lochhead, John H., Professor of Zoology, University of Vermont
Lorand, Laszlo, Associate Department of Physiology, Wayne University College of Medicine
Love, Warner E., Fellow of Johnson Foundation, University of Pennsylvania
Marsland, Douglas, Professor of Biology, Washington Square College
Mateyko, G. M., Fellow, Damon Runyon Mem. Fund, Washington Square College
Mauro, Alexander, Assistant Professor of Physiology, Yale University
Mazia, Daniel, Associate Professor of Zoology, University of California
Meinkoth, Norman A., Associate Professor of Biology, Swarthmore College
Merkel, Joseph R., Waksman-Merck Postdoctoral Fellow, Rutgers University
Metz, Charles B., Associate Professor of Zoology, University of North Carolina
Miura, Yoshiaki, Rockefeller Foundation Fellow, University of Tokyo, Japan
Moore, George M., Professor of Zoology, University of New Hampshire
Moore, John W., Biophysicist, Naval Medical Research Institute
Moul, Edwin T., Assistant Professor of Botany, Rutgers University
Nachmansohn, David, Associate Professor of Neurology, Columbia University

Nanninga, Ludo, Independent Investigator, Institute for Muscle Research
Neff, Robert J., Assistant Professor of Biology, Vanderbilt University
Nelson, Leonard, Instructor of Physiology, University of Nebraska
Odum, Howard Thomas, Assistant Professor, University of Florida
Osterhout, W. J. V., Member Emeritus, The Rockefeller Institute
Parpart, Arthur K., Professor and Chairman, Dept. of Biology, Princeton University
Pettibone, Marian H., Instructor, University of New Hampshire
Proctor, Nathaniel, Associate Professor of Biology, Morgan State College
Prosser, C. Ladd, Professor of Physiology, University of Illinois
Rieser, Peter, University of Pennsylvania
Roberts, John L., Instructor in Physiology, University of Massachusetts
Ronkin, R. R., Assistant Professor of Physiology, University of Delaware
Root, Walter S., Professor of Physiology, Columbia University
Rose, S. Meryl, Associate Professor of Zoology, University of Illinois
Roth, Jay S., Assistant Professor of Biochemistry, Hahnemann Medical College
Roys, Chester, Research Associate, Tufts College
Rugh, Roberts, Associate Professor of Radiology, Columbia University
Sandeen, Muriel I., Instructor in Zoology, Duke University
Schechter, Victor, Assistant Professor of Biology, City College of New York
Schleyer, Walter B., Research Associate, Columbia University
Sclufer, Evelyn, Graduate Student, University of Pennsylvania
Scott, Allan, Chairman, Department of Biology, Colby College
Scott, Dwight B. M., Associate in Biochemistry, Children's Hospital of Philadelphia
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College
Scott, George T., Professor, Oberlin College
Shanes, A. M., Head of Cellular Physiology, National Institute of Mental Health
Sartenaer, Paul J. M. J., Assistant at Institut Roy. des Sciences Nat., Belgium
Shaver, John R., Assistant Professor of Zoology, University of Missouri
Shedlovsky, Theodore, Associate Member, Rockefeller Institute
Silva, Paul Claude, Instructor of Botany, University of Illinois
Slifer, Eleanor H., Associate Professor of Zoology, State University of Iowa
Sols, Alberto, Research Fellow in Biochemistry, Washington University School of Medicine
Sonnenblick, B. P., Associate Professor of Biology, Rutgers University
Speidel, Carl C., Professor and Chairman of Anatomy, University of Virginia School of Medicine
Spratt, Nelson T., Professor of Zoology, University of Minnesota
Stanier, Roger, Professor of Bacteriology, University of California
Starr, Richard C., Assistant Professor of Botany, Indiana University
Steinbach, H. B., Professor of Zoology, University of Minnesota
Stokey, Alma G., Professor Emeritus of Plant Science, Mount Holyoke College
Strominger, Jack L., Senior Assistant Surgeon, National Institute of Arthritis and Metabolic Diseases
Stunkard, Horace W., Professor of Biology, New York University
Szent-Gyorgyi, Albert, Chief Investigator, Institute for Muscle Research
Szent-Gyorgyi, Andrew G., Independent Investigator, Institute for Muscle Research
Szent-Gyorgyi, Eve, Research Assistant, Institute for Muscle Research
Szent-Gyorgyi, Marta B., Research Assistant, Institute for Muscle Research
Taylor, Wm. Randolph, Professor of Botany, University of Michigan
Thomas, Lyell J., Instructor of Pharmacology, Woman's Medical College of Pennsylvania
Trinkaus, J. P., Assistant Professor of Zoology, Yale University
Troll, Walter, Assistant Director, May Institute for Medical Research
Tsuboi, Kenneth, Chemist, College of Physicians and Surgeons
Truji, Frederic I., Research Assistant in Biochemistry, Princeton University
Vernberg, F. John, Instructor of Zoology, Duke University
Villee, Claude A., Assistant Professor of Biochemistry, Harvard Medical School
Vincent, Walter S., Instructor in Anatomy, New York State University, School of Medicine at Syracuse

Wainio, Walter W., Associate Professor of Biochemistry, Rutgers University
Warner, Robert C., Assistant Professor of Chemistry, New York University College of Medicine
Webb, H. Marguerite, Assistant Professor, Goucher College
Whiting, P. W., Professor of Zoology, University of Pennsylvania
Wichterman, Ralph, Professor of Biology, Temple University
Wiercinski, Floyd J., Assistant Professor of Physiology, Hahnemann Medical College
Wilber, Charles, Medical Laboratories, Applied Physiology Branch
Wilson, Irwin B., Assistant Professor of Biochemistry, Columbia University
Wilson, Walter L., Assistant Professor of Physiology and Biophysics, University of Vermont College of Medicine
Witschi, Emil, Professor of Zoology, State University of Iowa
Wrinch, Dorothy, Lecturer in Physics, Smith College
Zotterman, Ygve, Professor, Veterinarske Hogskola, Stockholm, Sweden
Zweifach, Benjamin W., Associate Professor of Biology, New York University
Zwilling, Edgar, Associate Professor, University of Connecticut

4. Lalor Fellows, 1954

deVillafranca, George, Smith College
Hamer, Douglas, Cancer Research Laboratories, Birmingham, England
Huxley, A. F., Physiological Laboratory, Cambridge, England
Johnson, Thomas N., Michigan State College
Kalckar, Herman M., University of Copenhagen, Demark
Merkel, Joseph R., Rutgers University
Tsuboi, Kenneth, College of Physicians and Surgeons

Beginning Investigators, 1953

Chang, Joseph J., J. S. DeWitt Fellow in Biology, Princeton University
Chapman, Kent M., Teaching Assistant, University of Minnesota
Cohen, Melvin J., Graduate Research Zoologist, University of California
Collier, Jack R., Graduate Student, University of North Carolina
Couillard, Pierre, Graduate Student, University of Pennsylvania
Deck, J. David, Graduate Student, Princeton University
Dunn, Arnold, University of Pennsylvania
Eichhorn, John H., Research Assistant, Ohio State University
Ghiretti, Anna, Zoological Station, Naples, Italy
Gross, Paul C., Graduate Student, University of Pennsylvania
Hempling, Harold G., Graduate Student, Princeton University
Herr, Earl B., Graduate Student, University of Delaware
Howard, Robert S., Assistant Professor of Biological Sciences, University of Delaware
Jacob, Miriam, Teaching Assistant, New York University
Johnson, Thomas N., Instructor, Michigan State College
Kaye, Alvin M., Research Assistant, University of Pennsylvania
Kiebel, Geraldine, New York University
Landau, Joseph V., Damon Runyon Research Fellow, New York University
Maroney, Samuel P., Graduate Student, Duke University
Maynard, Donald M., Teaching Assistant, University of California
Moos, Carl, Graduate Student, Columbia University
Morrison, Thomas H., Graduate Student, Princeton University
Padykula, Helen A., Research Fellow, Harvard Medical School
Rafferty, Keen A., Jr., Teaching Assistant, University of Illinois
Ray, David T., Instructor of Zoology, Howard University
Rosenbaum, Leonard, Student, University of Delaware

Rosenberg, Robert, Research Fellow, Northwestern University
Rosenberg, Alburt M., Graduate Student, University of Pennsylvania
Shanklin, Douglas, New York State University College of Medicine at Syracuse
Skou, J. Christian, Assistant Professor, University of Denmark
Small, Jean E., Graduate Student, Brown University
Stephens, Grover C., Instructor in Biology, Brooklyn College
Stephenson, William K., Graduate Student, University of Minnesota
Thrasher, George Charles, University of Virginia School of Medicine
Tunik, Bernard D., Student, Columbia University

Library Readers, 1953

Angevine, Jay B., Jr., Graduate Assistant, Cornell University
Baker, Howard D., Associate Professor, Florida State University
Bauchau, Adrian G., Charge de Cours in Zoology, Facultás Universitaires, Namur, Belgium
Bloch, Robert, Research Associate, University of Pennsylvania
Bodansky, Oscar, Chief of Clinical Biochemistry, Sloan Kettering Institute
Cohen, Seymour S., Associate Professor of Physiol. Chem., University of Pennsylvania, Children's Hospital
Diller, Irene Corey, Associate Member of Institute, Institute for Cancer Research
Dixon, Frank J., Professor and Chairman, Dept. of Pathology, University of Pittsburgh School of Medicine
DuBois, Eugene F., Emeritus Professor of Physiology, Cornell University Medical College
Gabriel, Mordecai L., Assistant Professor, Brooklyn College
Gabrieli, Elemer, Research Fellow, Yale Medical School
Gaffron, Hans, Professor of Biochemistry, University of Chicago
Gruenberg, Benjamin C., Consultant, 100 Central Park South, New York 19
Henshaw, Paul S., Director of Research, Planned Parenthood Federation of America
Jones, Sarah R., Instructor of Zoology, Connecticut College
Kabat, Elvin A., Professor of Microbiology, College of Physicians and Surgeons
Karrer, Heinz E., Johns Hopkins University School of Hygiene and Public Health
Karush, Fred, Assistant Professor of Immunology, University of Pennsylvania
Kindred, James E., Professor of Anatomy, University of Virginia
Kinersly, Thorn, Research Fellow, Yale University School of Medicine
Kramer, Mollie P., Bibliographer, American Meteorological Society
Liang, Hsu-mu, Fellow of Cancer Research, Washington University
Love, Lois Coder, Chemical Biological Coordination Center, National Research Council
Lucke, Balduin, Professor of Pathology, University of Pennsylvania
McDonald, Sister Elizabeth Seton, Chairman, Dept. of Biology, College of Mount St. Joseph
Memhard, Allen R., 18 Crescent Road, Riverside, Connecticut
Morrison, David, Woods Hole, Mass.
Namias, Jerome, Chief, Extended Forecast Section, U. S. Weather Bureau
Ortiz, Evelina, Instructor in Zoology, University of Chicago
Racker, Efraim, Associate Professor of Biochemistry, Yale University
Reiner, John M., Assistant Professor of Biochemistry, Columbia University
Ross, Victor, Research Associate, College of Physicians and Surgeons
Schwartz, Martin, University Club, Madison, Wisconsin
Schwartzman, Gregory, Director, Dept. of Microbiology, Mount Sinai Hospital
Scott, Thomas F. M., Director of Research, Childrens Hospital of Philadelphia
Steigman, Alex J., Professor of Child Health, University of Louisville Medical School
Stern, Kurt G., Adjunct Professor of Biochemistry, Polytechnic Institute of Brooklyn
Sulkin, S. Edward, Professor and Chairman, Dept. of Bacteriology, Southwestern Medical School
Tondo, C. V., National Research Council of Brazil
Trotter, Mildred, Professor of Gross Anatomy, Washington University
Tsai, Tsuli, Fellow of Cancer Research, Washington University Medical School
Tyler, Albert, Professor of Embryology, California Institute of Technology

WILHELM, J. O., Director, Research Council of Ontario, Toronto, Canada
WOLLMAR, RICHARD, Technician, Seaplant Chemical Corporation
WYMENGA, HARRY G., Research Chemist, N. V. Organon, Oss, Holland
ZINN, DONALD J., University of Rhode Island

Research Assistants, 1953

ALLEN, MARJORY ANN, Indiana University
BAKER, GERALDINE M., Western Reserve University
BARNES, LOY J., University of Missouri
BENNETT, MIRIAM F., Northwestern University
BERMAN, MONES, Sloan Kettering Institute
BERNSTEIN, PAUL W., Washington University
BLANKENHORN, BRIGITTE, University of Maryland Medical School
BOOTH, ELIZABETH A., Columbia University
BORENFREUND, ELLEN, Columbia University
CHALFIN, DAVID, Princeton University
deTERRA, NOEL, Barnard College, Columbia University
DOSSEL, WILLIAM E., Johns Hopkins University
DRAKE, JOHN W., Yale University
FOX, HOWARD A., Colby College
FROST, JAMES L., Princeton University
GINTER, EILEEN L., Hahnemann Medical College
GLASSER, RICHARD L., University of Maryland Medical School
GNADE, ALLA, University of Pennsylvania
GRAVES, ROBERT C., Northwestern University
HINES, MARGARET N., Northwestern University
HOHNS, ELSIE D., New York University
ITO, SUSUMU, Western Reserve University
LACHANCE, LEO EMERY, North Carolina State College
LARIS, PHILIP C., Princeton University
LAUFER, WILMA, Tufts College
LECRONE, PATRICIA A., University of Delaware
LORIMER, ISABELLE, New York University
MCLAUGHLIN, JEAN, The Institute for Muscle Research
MAWE, RICHARD C., Princeton University
METZ, DELILAH B., New York University
PAULSEN, ELIZABETH, University of Vermont
PHILLPOTT, DELBERT, The Institute for Muscle Research
REICH, PETER, Harvard Medical School
RIMMLER, LUDWIG J., Syracuse University
RODGERS, FRANCES F., University of Pennsylvania
ROSENBLUTH, RAJA, Columbia University
SCHWARZ, NANCY L., University of Illinois
SERES, JOEL L., University of Delaware
STEINBERG, MALCOLM S., University of Minnesota
STONE, NOMI, Barnard College
SULLIVAN, ROBERT L., North Carolina State College
SUSSMAN, KARL E., University of Maryland Medical School
VAN BERGEIJK, WILLEM, State University of Iowa
VOORHEES, DAVID B., Yale University
WALTERS, PATRICIA, Lilly Research Laboratories
WATT, DONALD J., Columbia University
WESTFALL, JANE ANNE, University of North Carolina
WILCE, ROBERT T., University of Michigan
WYTTENBACH, CHARLES R., Indiana University
YOUNGS, LILLIAN M., University of North Carolina
ZIMMERMAN, ARTHUR M., New York University

Students, 1953

BOTANY

Bowes, Rev. David, Catholic University
Hillis, Llewellya, University of Michigan
Kanwisher, Joan, Woods Hole, Mass.
Marshall, Harold G., Western Reserve University
Dowling, John A., Harvard College
Noe, Frederick, Drew University
Rudolph, Emanuel, Washington University
Smith, Charlotte A., Vassar College
Stephenson, Phyllis C., University of Illinois
Stibritz, Mary G., Earlham College
Young, Richard S., University of North Carolina

ECOLOGY

Angevine, Emeline Midgett, Cornell University
Bond, Richard R., University of Wisconsin
Bronsweig, Ruth D., Bryn Mawr College
Greenburg, Michael J., Cornell University
Kuenzler, Edward J., Jr., University of Georgia
Perrotta, Carmie A., University of Wisconsin
Schreiman, David E., 107 Gifford Avenue, Jersey City, N. J.
Shaw, Evelyn S., American Museum of Natural History

EMBRYOLOGY

Ambellan, Elizabeth H., Columbia University
Beskind, Harry, Queens College
Borysko, Emil, Johns Hopkins University
Boulas, Stanley H., Brown University
Brailsford, Elizabeth S., Mt. Holyoke College
Clugston, Helen V., Oberlin College
Curley, Ellen L., Wellesley College
Dashman, Theodore, University of Illinois
Denny, David, Dillard University
Doolin, Paul F., University of Illinois
Duncan, James T., Wabash College
Erickson, Joan, Radcliffe College
Gallagher, John C., Yale University
Grabowski, Casimer T., Johns Hopkins University
Hegan, Nancy A., Pennsylvania College for Women
Hilfer, Saul Robert, Queens College
Hodge, Mary H., Simmons College
Jeffries, William B., University of North Carolina
LeTourneau, David J., Wesleyan University
McKinnell, Robert G., Springfield 4, Missouri
McMurray, Virginia M., State University of Iowa
Merrill, Arthur S., Harvard University
Milkman, Roger D., Harvard University
Park, Helen D., George Washington University
Rudin, Robert, University of North Carolina
Spiegelman, Irvin Morton, Amherst College
Swartz, Frank J., Western Reserve University
Telfer, William H., Harvard University
Tucker, Rev. Eugene L., Loyola College
Wessells, Norman K., Yale University

PHYSIOLOGY

Bakemeier, Richard F., University of Rochester
Behrman, Edward T., University of California
Bibring, Thomas, Stanford University
Birks, Richard I., McGill University
Brill, Arthur S., University of Pennsylvania
Cascarano, Joseph, New York University
Child, Frank M., Amherst College
Febvre, Henri, The Rockefeller Institute
Geller, David M., Harvard University
Gonzalez, Elsa L., Columbia University
Haggis, Alex J., University of Rochester
Harris, Joseph D., Purdue University
Harvey, Harriet R., University of Oklahoma
Kowal, Jerome, Johns Hopkins Medical School
Lambert, Francis L., Harvard University
Leventhal, David E., University of Illinois
Litman, Rose M., Indiana University
Mann, Jay D., Brooklyn College
Mills, Kenneth S., University of Texas
Olson, John M., University of Pennsylvania
Potter, David D., Harvard University
Rapoport, Stanley I., Princeton University
Rieck, Alvin F., Marquette University
Roeder, Martin, University of North Carolina
Roter, Ellen Celia, University of Illinois
Rothschild, Hanna A., University of Sao Paulo, Brazil
Schneiderman, Howard A., Cornell University
Solano, Sister Francis, Nazareth College
Straus, Mark, 1901 Bedford Avenue, Brooklyn, N. Y.
Theophilus, Victoria, Vassar College
Wolbach, Robert A., Cornell University School of Medicine

INVERTEBRATE ZOOLOGY

Ashton, Francis T., Swarthmore College
Atz, James W., New York Zoological Society
Barsa, Mary C., Fordham University
Bendix, Selina, University of California
Bierman, Sheila, Cornell University
Brown, Paul L., University of Illinois
Cory, Robert L., University of Delaware
Cumings, Edwin H., DePauw University
Curry, George M., Harvard University
Dowling, Mary C., Seton Hill College
Drake, John W., Yale University
Eisenman, Joseph, City College of New York
Fraser, Elizabeth F., Bedford College, London, England
Fraser, Jean F., Bedford College, London, England
Frost, David, New York University
Green, Donald M., Oberlin College
Green, Paul B., University of Pennsylvania
Hadley, Doris S., George Washington University
Hagen, Lynette M., Brothers College of Drew University
Hartshorne, James M., Cornell University
Harvey, William R., Harvard University
Haubrich, Robert R., University of Florida
Heafitz, Morton H., Dartmouth College
Hickok, John F., Cate School, California

Lappano, Eleanor R., Fordham University
Linder, Harris J., Cornell University
Marko, Anita R., Adelphi and Hofstra Colleges
McDaniel, Harriet C., Brooklyn College
McKernan, Sally A., University of Kansas
McNamara, Dorris, New York University
Messinger, Eli Charles, Lafayette College
Mizell, Merle, University of Illinois
Moffatt, Jane L., George Washington University
Passaglia, Martin A., St. Louis University
Patton, Wendell K., Hamilton College
Pierro, Louis, Marquette University
Pryor, Carlon W., University of Kansas
Reed, Robert R., Purdue University
Renner, John H., Dartmouth College
Richards, Marie L., Maryville College
Roberts, Jane C., University of Massachusetts
Roslansky, John D., University of California
Rowe, Edward C., Wesleyan University
Runyon, Miriam, Vassar College
Sargent, Lydia M., Knox College
Schlick, Roberta A., Knox College
Schlueter, Edgar A., University of Wisconsin
Senterfit, Laurence B., Johns Hopkins Hospital
Shriner, Joan, Mount Holyoke College
Skinner, Dorothy M., Tufts College
Stevenson, Joseph R., Oberlin College
Sullivan, Robert L., North Carolina State College
vanLaer, Helen R., Johns Hopkins University
Warwick, Anne Charlotte, Johns Hopkins University
Wendt, Richard H., University of Rochester

5. Tabular View of Attendance, 1949–1953

	1949	*1950*	*1951*	*1952*	*1953*
Investigators—Total	344	338	303	300	310
Independent	193	198	186	172	176
Under Instruction	52	43	28	38	37
Library Readers	55	48	37	49	46
Research Assistants	44	49	52	47	51
Students—Total	128	126	124	123	136
Zoology	55	55	55	55	55
Embryology	31	29	27	23	30
Physiology	27	27	29	27	31
Botany	15	13	13	11	11
Ecology				7	9
Total Attendance	472	444	427	423	446
Less persons registered as both students & investigators	2			2	
	470	444	427	421	446
Institutions Represented—Total	155	156	158	149	155
By Investigators	114	114	115	92	90
By Students	68	67	43	57	65
Schools and Academies Represented					
By Investigators	1	2	1	1	
By Students				3	1
Foreign Institutions Represented					
By Investigators	6	6	8	7	15
By Students	3	2	3	2	6

6. Cooperating and Subscribing Institutions, 1953

Cooperating Institutions

Amherst College
Brooklyn College
Brown University
Bryn Mawr College
California Institute of Technology
Carnegie Embryological Laboratory
Children's Hospital of Philadelphia
Colby College
College of Mt. Joseph-on-the-Ohio
College of Physicians and Surgeons
Columbia University
Cornell University
Drew University
Duke University
Elmira College
Emory University
Fordham University
George Washington University
Hahnemann Medical School
Harvard University
Harvard University Medical School
Eli Lilly and Company
Marquette University
Massachusetts General Hospital
Morgan State College
Mount Holyoke College
National Institute of Arthritis and Metabolic Diseases
National Science Foundation
New York University College of Medicine
New York University, Heights
New York University, Washington Square College
North Carolina State College of Agriculture and Engineering
Northwestern University
Oak Ridge Institute for Nuclear Studies
Oberlin College
Princeton University
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Seaplant Chemical Corporation
Sloan Kettering Institute
Southwestern Medical College
State University of Iowa
State University of New York, College of Medicine at Syracuse
Temple University
Tufts College
University of Chicago
University of Connecticut
University of Delaware
University of Illinois
University of Maryland School of Medicine
University of Massachusetts
University of Michigan
University of Minnesota
University of New Hampshire
University of North Carolina
University of Pennsylvania
University of Pennsylvania Medical School
University of Rochester
University of Vermont Medical School
University of Virginia
University of Wisconsin
Vassar College
Washington University School of Medicine
Wellesley College
Wesleyan University
Western Reserve University School of Medicine
Yale University

Subscribing Institutions

American Bureau of Medical Aid to China
Belgian American Educational Foundation
City College of New York
Cleveland Audubon Society
Dillar University
Hamilton College
Indiana University
Loyola College
May Institute for Medical Research
Mount Sinai Hospital
Ohio State College
Pennsylvania College for Women
Radcliffe College
University of California
University of Kentucky
University of Louisville Medical School
University of Mississippi
University of Nebraska
University of Pittsburgh
Vanderbilt University
Wabash College
Washington University
Women's Medical College of Pennsylvania

7. Evening Lectures, 1953

July 3	Hermann Kalckar	"Nucleotides, their synthesis and their functions."
July 10	Horace W. Stunkard	"Parasitism, a phase of marine biology."
July 17	E. S. G. Barron	"The importance of sulfhydryl groups in biology and medicine."
July 24	H. Keffer Hartline	"Electrical activity of visual receptors and optic nerve fibers."
July 31	Robert Briggs	"The problem of nuclear differentiation in embryonic development."
August 7	P. F. Scholander	"Homiothermism in relation to climate."
August 10	A. F. Huxley	"Muscle fibers under the microscope."
August 14	Ernest Pollard	"The physics of viruses."
August 21	Leigh E. Chadwick	"Insect cholinesterases."

8. Tuesday Evening Seminars, 1953

June 20	Dorothy Wrinch	"New evidence regarding the structure of ribonuclease."
	W. S. Vincent	"An approach to the problem of DNA constancy."
	Daniel Mazia	"Chemical and electron microscopic observations on the isolated mitotic apparaus."
July 7	Warner Hammond	"The neuronal derivatives of the neural crest in the chick."
	Sears Crowell	"The regression-replacement cycle of hydranths in *Obelia* and *Campanularia*."
	Motion Picture:	F. S. Hammett's time lapse study of the development and regression of hydranths of Obelia.
July 14	Harold G. Hempling	"Potassium and sodium movements in rabbit polymorphonuclear leukocytes."
	L. Lorand	"Clotting of fibrinogen as a problem of protein chemistry."
	Max A. Lauffer, A. Buzzell and D. Trkula	"Thermal inactivation of a bacterium-bacteriophage complex at various stages of development."

July 21

Irwin B. Wilson .... "Some features of the proteins associated with the generation of bioelectricity."

Mario Altamirano .... "The relation between the chemical structure of some compounds and their effect upon bioelectric phenomena."

Y. Zotterman .... "The effect of acetylcholine and related compounds on sensory organs."

July 28

James W. Green and F. X. Wazeter .. "Glycolysis and potassium loss in rabbit red cells exposed to ethyl and propyl carbonate."

F. R. Hunter and Alice S. Baker .... "A study of the oxidative activity of rabbit reticulocytes."

George T. Scott and Hugh Hayward .. "Metabolic factors influencing the distribution of sodium and potassium ions in the green alga *Ulva lactuca*."

August 4

Stephen Hajdu .... "On the mechanism of staircase and contracture in ventricular muscle."

C. Ladd Prosser .... "Conduction in non-striated muscles."

Abraham M. Shanes .... "Structural and *in vitro* diffusion characteristics of intact and 'desheathed' sciatic nerves from the toad (*B. merinus*) and the bullfrog (*R. catesbiana*)."

August 11

Jay S. Roth .... "Some observations on the ribonuclease activity of separated cell particulates."

Walter Troll and Sol Sherry .... "Synthetic substrates for fibrinolysin (plasmin) and thrombin."

August 18

Malcolm Steinberg .... "Studies on the mechanism of physiological dominance in Tubularia."

Walther Hild .... "Elaboration of so-called posterior lobe hormone in the hypothalamus."

Y. Zotterman .... "Primary polydipsia in goats (motion picture)."

9. Members of the Corporation, 1953

1. Life Members

Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York.
Billings, Mr. R. C., 66 Franklin Street, Boston, Massachusetts.
Budington, Dr. R. A., Box 954, Winter Park, Florida.
Brodie, Mr. Donald M., 522 Fifth Avenue, New York 18, New York.
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania.
Cole, Dr. Leon J., College of Agriculture, Madison, Wisconsin.
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri.
Dungay, Dr. Neil S., Carleton College, Northfield, Minnesota.
Goldfarb, Dr. A. J., College of the City of New York, New York City, New York.
Jackson, Mr. Charles C., 24 Congress Street, Boston, Massachusetts.

Jackson, Miss M. C., 88 Marlboro Street, Boston, Massachusetts.
King, Mr. Charles A.
King, Dr. Helen D., Wistar Inst. of Anatomy and Biology, Philadelphia, Pennsylvania.
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Maryland.
Lowther, Dr. Florence deL., Barnard College, New York City, New York.
MacNaught, Mr. Frank M., Woods Hole, Massachusetts.
Means, Dr. J. H., 15 Chestnut Street, Boston, Massachusetts.
Moore, Dr. George T., Missouri Botanical Gardens, St. Louis, Missouri.
Moore, Dr. J. Percy, University of Pennsylvania, Philadelphia, Pennsylvania.
Noyes, Miss Eva J.
Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
Riggs, Mr. Lawrason, 74 Trinity Place, New York 6, New York.
Scott, Dr. Ernest L., Columbia University, New York City, New York.
Sears, Dr. Henry F., 86 Beacon Street, Boston, Massachusetts.
Shedd, Mr. E. A.
Waite, Prof. F. C., 144 Locust Street, Dover, New Hampshire.
Wallace, Louise B., 359 Lytton Avenue, Palo Alto, California.
Warren, Dr. Herbert S., 610 Montgomery Avenue, Bryn Mawr, Pennsylvania.
Young, Dr. B. P., Cornell University, Ithaca, New York.

2. Regular Members

Abell, Dr. Richard G., 7 Peter Cooper Road, New York City, New York.
Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts.
Addison, Dr. W. H. F., 286 East Sidney Avenue, Mount Vernon, New York.
Adolph, Dr. Edward F., University of Rochester School of Medicine and Dentistry, Rochester, N. Y.
Albaum, Dr. Harry G., Biology Department, Brooklyn College, Brooklyn, New York.
Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota.
Allee, Dr. W. C., 114 Leigh Hall, University of Florida, Gainesville, Florida.
Alscher, Dr. Ruth, Dept. of Physiology, Manhattanville College of the Sacred Heart, Purchase, New York.
Amberson, Dr. William R., Dept. of Physiology, University of Maryland School of Medicine, Baltimore, Md.
Anderson, Dr. Rubert S., Medical Laboratories, Army Chemical Center, Maryland.
Anderson, Dr. T. F., University of Pennsylvania, Philadelphia, Pennsylvania.
Armstrong, Dr. Philip B., State University of New York, College of Medicine, Syracuse 10, New York.
Atwood, Dr. Kimball C., 68½ Outer Drive, Oak Ridge, Tennessee.
Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts.
Ayers, Dr. John C., Department of Oceanography, Cornell University, Ithaca, New York.
Baitsell, Dr. George A., 54 Hill House Ave., Connecticut.
Baker, Dr. H. B., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.

Ball, Dr. Eric G., Dept. of Biological Chemistry, Harvard University Medical School, Boston 15, Massachusetts.

Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire.

Bard, Dr. Philip, Johns Hopkins Medical School, Baltimore, Maryland.

Barron, Dr. E. S. G., Department of Medicine, University of Chicago, Chicago, Illinois.

Barth, Dr. L. G., Department of Zoology, Columbia University, New York City, New York.

Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois.

Beams, Dr. Harold W., Department of Zoology, State University of Iowa, Iowa City, Iowa.

Beck, Dr. L. V., Dept. of Physiology and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania.

Beers, Dr. C. D., University of North Carolina, Chapel Hill, North Carolina.

Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, Louisiana.

Bertholf, Dr. Floyd M., College of the Pacific, Stockton, California.

Bevelander, Dr. Gerrit, New York University School of Medicine, New York City, New York.

Bigelow, Dr. Henry B., Museum of Comparative Zoology, Harvard University, Cambridge, Mass.

Bigelow, Dr. Robert P., Massachusetts Institute of Technology, Cambridge, Massachusetts.

Bishop, Dr. David W., Dept. of Embryology, Carnegie Inst. of Washington, Baltimore 5, Maryland.

Blanchard, Dr. K. C., Johns Hopkins Medical School, Baltimore, Maryland.

Bloch, Dr. Robert, Research Associate in Botany, University of Pennsylvania, Philadelphia, Pa.

Blum, Dr. Harold F., Department of Biology, Princeton University, Princeton, New Jersey.

Bodansky, Dr. Oscar, Dept. of Biochemistry, Sloan-Kettering Division, Cornell University Medical College, New York City, New York.

Bodine, Dr. J. H., Department of Zoology, State University of Iowa, Iowa City, Iowa.

Bodian, Dr. David, Department of Epidemiology, Johns Hopkins University, Baltimore 5, Maryland.

Boell, Dr. Edgar J., Yale University, New Haven, Connecticut.

Boettiger, Dr. Edward G., Department of Zoology, University of Connecticut, Storrs, Conn.

Bonner, Dr. John T., Department of Biology, Princeton University, Princeton, New Jersey.

Borei, Dr. Hans G., Dept. of Zoology, University of Pennsylvania, Philadelphia, Pa.

Bradley, Dr. Harold C., 2639 Durant Avenue, Berkeley 4, California.

Bronfenbrenner, Dr. J. J., 1703 Reynolds Road, Winston-Salem, North Carolina.

Bronk, Dr. Detlev W., Rockefeller Inst. for Medical Research, New York 21, N. Y.

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Brown, Dr. Frank A., Jr., Department of Biological Sciences, Northwestern University, Evanston, Illinois.

Brown, Dr. Dugald E. S., Department of Zoology, University of Michigan, Ann Arbor, Michigan.

Brownell, Dr. Katherine A., Ohio State University, Columbus, Ohio.

Buck, Dr. John B., Laboratory of Physical Biology, National Institutes of Health, Bethesda, Md.

Buckingham, Miss Edith N., Sudbury, Massachusetts.

Bullington, Dr. W. E., Randolph-Macon College, Ashland, Virginia.

Bullock, Dr. T. H., University of California, Los Angeles 24, California.

Burbanck, Dr. William D., Box 834, Emory University, Georgia.

Burdick, Dr. C. Lalor, The Lalor Foundation, Lancaster Pike and Old Baltimore Road, Wilmington, Delaware.

Burkenroad, Dr. M. D., Institute of Marine Science, Port Arkansas, Texas.

Butler, Dr. E. G., Princeton University, Princeton, New Jersey.

Cameron, Dr. J. A., Baylor College of Dentistry, Dallas, Texas.

Cannan, Dr. R. K., National Research Council, 2101 Constitution Avenue, N.W., Washington, D. C.

Cantoni, Dr. Giulio, Department of Pharmacology, Western Reserve University, Cleveland 9, Ohio.

Carlson, Dr. A. J., Department of Physiology, The University of Chicago, Chicago 37, Illinois.

Carothers, Dr. E. Eleanor, 134 Avenue C., East Kingman, Kansas.

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Carson, Miss Rachel, 204 Williamsburg Drive, Silver Spring, Maryland.

Carver, Dr. Gail L., Mercer University, Macon, Georgia.

Cattell, Dr. McKeen, Cornell University Medical College, 1300 York Avenue, New York City, N. Y.

Cattell, Mr. Ware, Cosmos Club, Washington 5, D. C.

Chambers, Dr. Edward, Dept. of Anatomy, Univ. of Oregon Medical School, Portland, Ore.

Chambers, Dr. Robert, University Club, Portland, Oregon.

Charles, Dr. Donald R., Dept. of Zoology, Division of Biological Sciences, University of Rochester, Rochester 3, New York.

Chase, Dr. Aurin M., Department of Biology, Princeton University, Princeton, New Jersey.

Cheney, Dr. Ralph H., Biology Department, Brooklyn College, Brooklyn 10, New York.

Child, Dr. C. M., Jordan Hall, Stanford University, Stanford, California.

Churney, Dr. Leon, Dept. of Physiology, Louisiana State University School of Medicine, New Orleans, La.

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Clark, Dr. A. M., Department of Biology, University of Delaware, Newark, Delaware.

Clark, Dr. E. R., The Wistar Inst., Woodland Avenue and 36th Street, Philadelphia 4, Penn.

Clark, Dr. Leonard B., Department of Biology, Union College, Schenectady, New York.

Clarke, Dr. George L., Harvard University, Biological Laboratory, Cambridge 38, Massachusetts.

Cleland, Dr. Ralph E., Indiana University, Bloomington, Indiana.

Clement, Dr. A. C., Department of Biology, Emory University, Emory, Georgia

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VI. REPORT OF THE LIBRARIAN

1953

During 1953, the number of journals currently received totalled 1503 (67 new). There were 461 (14 new) Marine Biological Laboratory subscriptions; 600 (21 new) exchanges and 163 (15 new) gifts; 73 (6 new) were Woods Hole Oceanographic Institution subscriptions; 173 (5 new) were exchanges and 33 (6 new) were gifts.

The Marine Biological Laboratory purchased 39 books, received 51 complimentary copies (7 from authors and 44 from publishers) and 14 miscellaneous donations. The Woods Hole Oceanographic Institution purchased 23 titles and received 7 gifts. The total number of books placed on the shelves amounted to 134. The Marine Biological Laboratory completed by purchase 7 journal sets and partially completed 9 sets. The Woods Hole Oceanographic Institution completed 3 back sets and partially completed 4 sets. Volumes and numbers were received by gift and by exchange which partially completed 9 sets.

The reprint collection was increased by 5643 papers, 2464 being of current issue.

Forty-seven titles were borrowed on inter-library loan and 125 were sent to out-of-town libraries. The sale of duplicate material contributed $263.45 to the Laboratory's general fund. The microfilm service has not as yet been resumed.

The Library gratefully acknowledges the following gifts: from Dr. Paul S. Galtsoff, his card bibliography of references pertaining to the "Gulf of Mexico—Its Origin, Waters and Marine Life," as well as numbers and volumes which further complete 10 Russian titles; a set of the Josiah Macy Foundation publications from the Foundation; a current subscription to "Scientific American" from Dr. Ross G. Harrison, Jr.; the early volumes of "Nucleonics" from Mr. Jack Hagstrom; and reprint collections from Dr. and Mrs. P. W. Whiting, Smith College Library, Dr. L. J. Milne, Dr. Ursula M. Höber, and the U. S. Army Chemical Center in Maryland.

At the end of the year, the Library contained 63,276 bound volumes and 188,584 reprints.

The Librarian wishes to express her appreciation to Dr. Wm. Randolph Taylor for the help and advice so willingly given during his term as Chairman of the Library Committee.

Respectfully submitted,
Deborah L. Harlow,
Librarian.

PHASE–CONTRAST AND ELECTRON MICROSCOPE STUDIES ON THE NEBENKERN, A MITOCHONDRIAL BODY IN THE SPERMATIDS OF THE GRASSHOPPER

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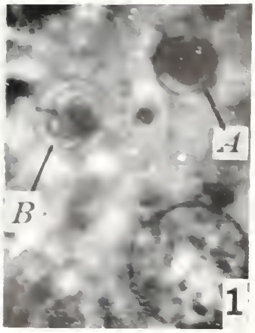
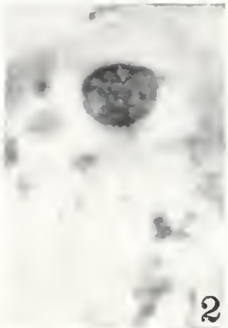
Attempts to demonstrate a structure within mitochondria which might be correlated with their function have been the object of several investigations: (1) In the germ cells of certain insects the filamentous mitochondria are composed of two distinct parts, an outer chromophilic and an inner chromophobic (Gatenby, 1917; Bowen, 1922). (2) In electron micrographs certain mitochondria show areas of unequal electron density (Claude and Fullman, 1945). (3) Sometimes the particles appear to be held together within a lattice framework (Pease, Baker and Warren, 1950). (4) Other mitochondria show cross striations (Pease and Baker, 1950), while still others possess a system of internal ridges, or cristae, that protrude perpendicularly from the inside surface of the membrane toward the interior of the mitochondrion (Palade, 1952, 1953). Similar structures have been observed to extend across the mitochondria and to be composed of double membranes (Sjöstrand, 1953). (5) In contrast to the above, certain other mitochondria display longitudinally oriented internal filaments or lamellae (Glimstedt and Lagerstedt, 1953; Beams and Tahmisian, 1954). Mitochondria of this type show birefringence in their longitudinal axis (Monné, 1948). In addition, variation in mitochondrial form, size and structure has been shown to occur in cells of animals exposed to pathological or experimental conditions. The demonstration of a limiting membrane at the surface of the mitochondria by Dalton *et al.* (1949) has, for the most part, been confirmed (Palade, 1952; Sjöstrand, 1953 and others).

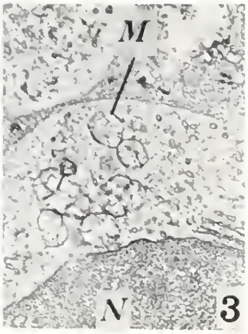
Since the nebenkern in the spermatids of certain animals has been shown to be a mitochondrial body, *i.e.,* composed of fused mitochondria (von la Valette St. George, 1886; Meves, 1900; Lewis and Robertson, 1916; Payne, 1916, 1926; Gatenby, 1917; Bowen, 1922; Pollister, 1930; Johnson, 1931 and others), it seemed of interest to attempt by aid of the electron microscope an analysis of its ultramicroscopic structure and to compare the structure thus revealed with that cited above for other mitochondria. Limited observations have also been made on the nebenkern of living cells by aid of the phase-contrast microscope, and the structure thus revealed compared to that of comparable stages described for it in fixed and stained preparations.

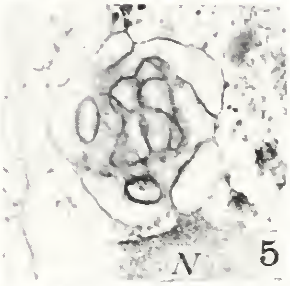
MATERIAL AND METHODS

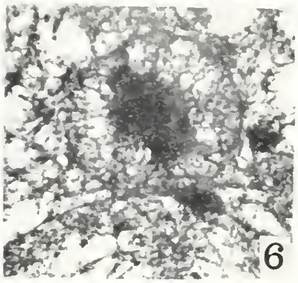
The material for this study consists of the testes of grasshopper nymphs, *Melanoplus differentialis.* After removing the testis from the body, it was placed in Bělař solution and dissected free of fat and connective tissue. About three tubules were then placed in a few drops of Bělař solution in the lower half of a roto-compressor

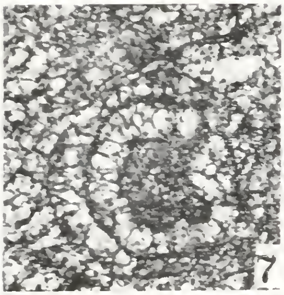


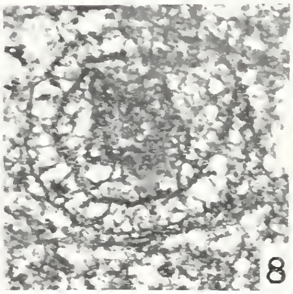


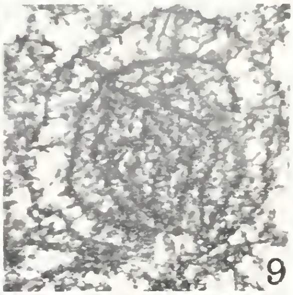


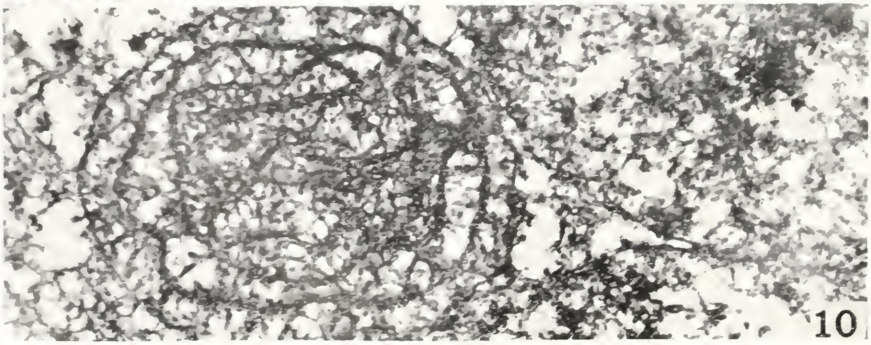












All figures in Plates I to III (except Figs. 1 and 2) are electron micrographs. Figures 1 and 2 are phase-contrast microscope pictures of living cells.

PLATE I

FIGURE 1. Phase-contrast microscope picture of two nebenkern. The one at A is in a relatively early stage of development and shows a comparatively dense core surrounded by a vacuolar layer and a delimiting membrane or lamella. The one at B is a mature stage and shows

where their tips were cut to allow the cells of the tubules to be displaced into the surrounding fluid. The top of the micro-compressor was then placed in position and tightened until the cells showed slight compression when viewed through the Leitz phase-contrast microscope.

The fixatives used for electron microscope studies were Champy's, 2% osmic acid, and buffered 2% osmic acid (pH 7.35). Tubules from fixed testes were embedded in methacrylate, sectioned at 0.1 μ and studied by aid of an R. C. A. model E. M. U. electron microscope.

RESULTS

Phase-contrast microscope observations.

Preliminary observations on the structure of the nebenkern in different stages of development with the phase-contrast microscope showed it to be essentially the same as that seen in fixed and stained preparations. For this reason, an extensive study on the structure of the nebenkern by this method was not attempted. However we have included two figures of the phase-contrast microscope part of this study, each of which resembles closely similar stages figured by Bowen (1922) and others. Figure 1A, according to Bowen (1922) is a relatively early stage in the differentiation of the nebenkern. It will be noted that at this stage a light area is evident near the periphery, which is presumably being differentiated by a vacuolization process from the central sponge region. This process continues until a fully differentiated nebenkern with concentric lamella of about 0.50 μ in width and separated by inter-lamellar spaces is formed (Fig. 1B). Here also is seen a plate or filament arranged at right angles to the concentric lamellae; the latter are probably associated with the division mechanism occurring in the nebenkern of other spermatids. Figure 2 shows a vacuolated condition of the nebenkern which is believed to be a relatively late stage in its sequence.

As will be noted below, it is difficult to correlate all the structure of the nebenkern seen with the phase-contrast and light microscopes with that observed in electron micrographs.

Electron microscope observations.

Out of approximately 150 electron micrographs of spermatids showing nebenkerns we have attempted to arrange in consecutive series those which seem to show pro-

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a series of concentric lamellae alternating with layers of matrix. A relatively dense membrane extends across its axis. × 1800.

FIGURE 2. Phase-contrast picture of a vacuolated nebenkern. × 1800.

FIGURE 3. Group of mitochondria (M) that are apparently fusing in the initial stages of the formation of the nebenkern. The nucleus is shown at N. Buffered 2% osmic acid fixation. × 5000.

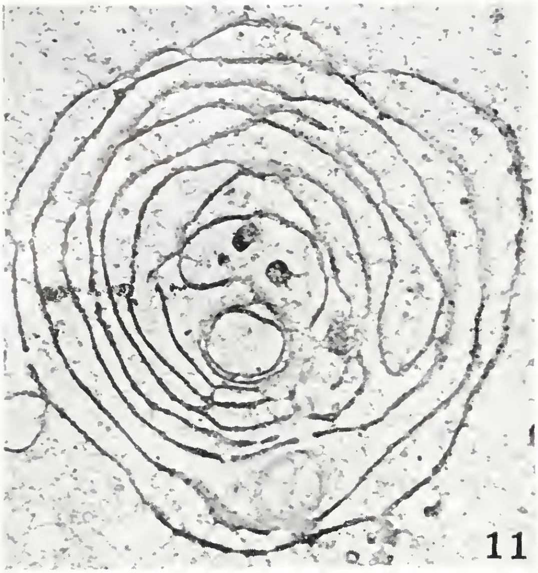
FIGURES 4 AND 5. Early stages in the formation of the nebenkern showing more extensive fusion of particles and the initial stages in formation of the concentric lamellae. In Figure 5 the nucleus is shown at N. Buffered 2% osmic acid fixation. × 5000.

FIGURE 6. Section through the peripheral region and across the long axis of a mature nebenkern. Champy's fixation. × 5000.

FIGURES 7 AND 8. Sections through successively deeper levels of the mature nebenkern revealing its internal structure. Champy's fixation. × 8000.

FIGURE 9. A mature stage of nebenkern showing 4 to 5 concentric lamellae. Champy's fixation. × 10,000.

FIGURE 10. Obliquely sectioned nebenkern. Some of the lamellae in upper half of figure seem double. Champy's fixation. × 17,000.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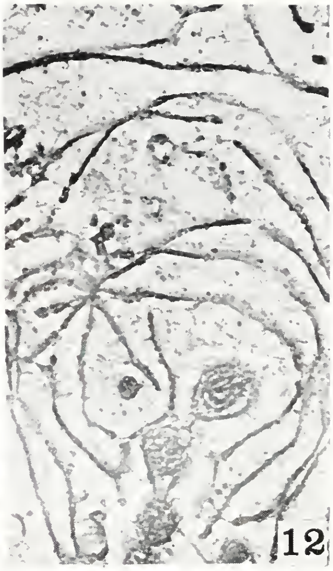
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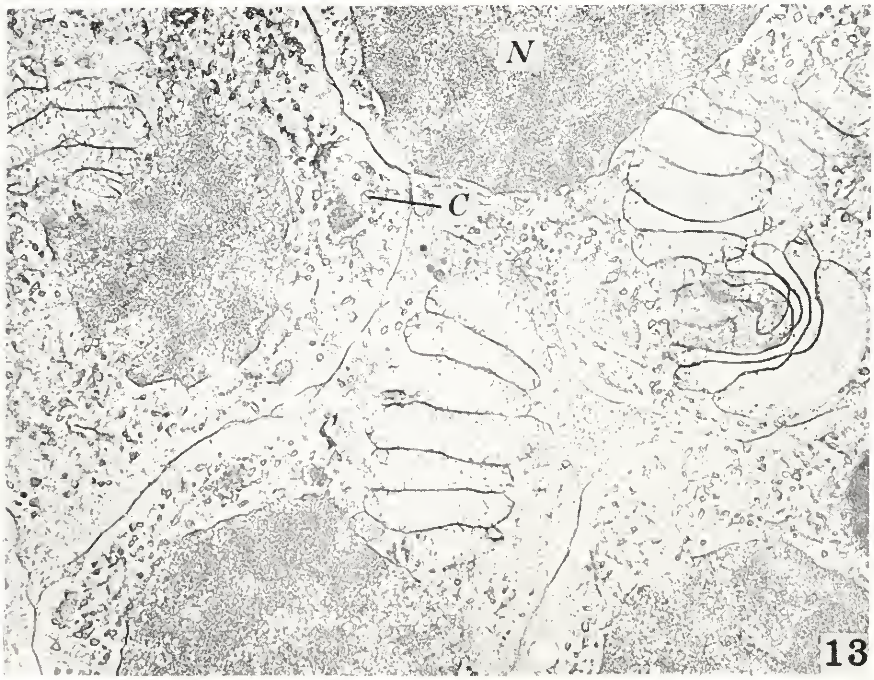

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Plate II

Figure 11. Cross section of mature nebenkern. Here the lamellae measure about 400 Å in thickness. The interlamellar matrix shows no special structure. Buffered 2% osmic acid fixation. × 16,500.

gressive stages in structural differentiation, the peak of which is followed by dedifferentiation in the transforming spermatid. Such an arrangement is not an easy task, even when studied in light microscope preparations (Bowen, 1922), and it becomes an even more difficult one when studied at the ultramicroscopic level.

Immediately following division of the second spermatocyte the somewhat swollen and vesicular mitochondria collect at one side of the nucleus in the newly formed spermatids (Fig. 3M). As development proceeds, they seem to fuse (Figs. 4, 5, and 18A), giving rise to elongated vesicles, the surfaces of which appear to form the dense concentric laminated structures; the remaining part gives rise to the less dense inter-lamellar matrix (Figs. 9, 10, 11, and 12).

As will be noted below, when completely differentiated the nebenkern is composed of a series of concentric lamellae separated by inter-lamellar matrix. Figures 6 to 10 seem to show a series of transverse sections at different depths through the completely differentiated nebenkern. Figure 6 appears to represent a section through the peripheral region and across the longitudinal axis. If the lamellae are numbered from the outside inward, Figure 6 represents a section in which the knife passed across the first, or outer, lamella and through, or just above, the second lamella. Similar, but deeper, sections are noted in Figures 7 and 8 where they are through the level of the third and fourth lamellae, respectively. When a more central section is made through the nebenkern, 6 to 8 concentric lamellae are often evident (Figs. 9 to 11). In Figure 9 the section is through the longitudinal axis of the nebenkern; it is in this axis that it will eventually divide (Figs. 14, 15) and elongate (Fig. 16), giving rise to part of the structure in the middle piece of the sperm. The lamellae of the nebenkern measure about 400 Å in thickness and the inter-lamellar spaces about 1800 to 3000 Å in thickness. Figure 10 is an oblique section through the nebenkern and is interesting because some of its lamellae appear to be composed of double membranes. When viewed in cross section the concentric lamellae of the nebenkern are approximately eight in number (Fig. 11). Little structure is observed within the inter-lamellar matrix which could be used to differentiate it from the surrounding cytoplasm. A section through one pole of the nebenkern is seen in Figure 12, while Figure 13 shows sections through the sides of what seem to be developing stages. The scattered vesicular bodies in the cytoplasm of these cells are probably diffuse basophilic substance or what Porter (1953) has called endoplasmic reticulum (Fig. 13C).

In older spermatids, those in the initial stages of transformation into spermatozoa, the nebenkern divides into two parts (Figs. 14, 15, and 16). At this time it loses its lamellar structure, becomes somewhat vesicular, and elongates into the middle piece of the developing sperm (Fig. 16). A few electron-opaque bodies are still evident within the halves of the nebenkern but the lamellae have disappeared, leaving a comparatively structureless matrix. A centriole may be observed here at

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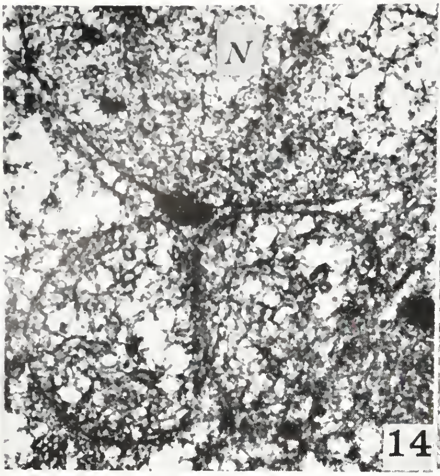
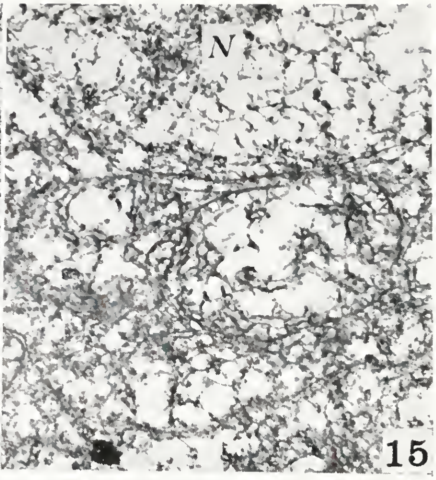
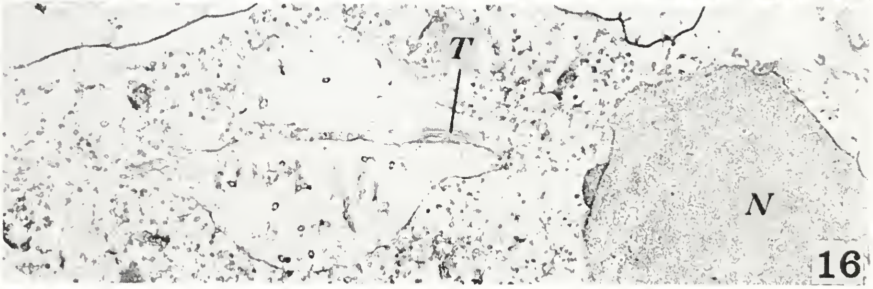
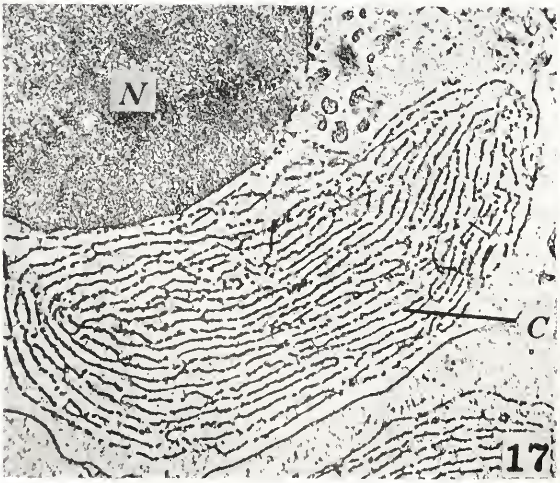
FIGURE 12. Section near one pole of nebenkern, revealing the arrangement of lamellae. Buffered 2% osmic acid. × 17,000.

FIGURE 13. Longitudinal section through side of developing nebenkern revealing outer lamellae. The nucleus is shown at N and the endoplasmic reticulum at C. Buffered 2% osmic acid fixation. × 5000.









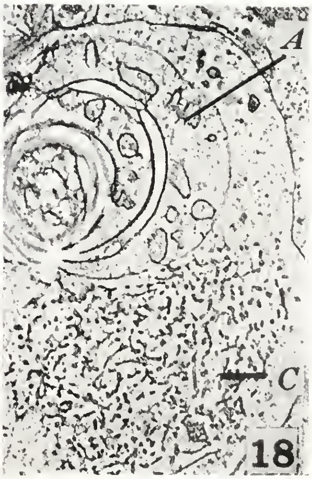


Plate III

Figures 14 and 15. Initial stages in sperm development, in which the nebenkern has lost its lamellae and divided, preparatory to moving into the middle piece. The nucleus is seen at N. Champy's fixation. Figure 14, × 10,000; Figure 15, × 5000.

the base of the nucleus, and a portion of the tail filament (T) is revealed between the halves of the elongating nebenkern.

Certain unidentified bodies were observed in the electron micrographs of the germ cells of the grasshopper (Figs. 17C and 18C). We believe these to be basophilic bodies which have been formed by a fusion of scattered basophilic material like that present in the cells of Figure 13C. These bodies show filaments or lamellae about 600 Å in width which are probably composed of double membranes. Between the filaments or lamellae are relatively structureless layers of matrix which measure on the average about 2170 Å in width. In Figure 18C the lamellae are seen in cross section. It is clear that this body is not a part of the nebenkern, since both bodies are sometimes revealed side by side in the same cell (Figs. 18A, C).

Discussion

Mitochondrial material is known to exist in many different forms such as granules, rods, filaments and vesicles; it is also known to be the locus of many important enzymatic functions. (For an excellent review of the morphology and biochemistry of mitochondria the reader is referred to the symposium on this subject appearing in the *J. of Histochem. and Cytochem.*, vol. 1, pp. 179 to 281, 1953.)

One of the most interesting types of mitochondrial bodies is the nebenkern which exists in the spermatids of certain insects (von la Valette St. George, 1886; Meves, 1900; Lewis and Robertson, 1916; Gatenby, 1917; Bowen, 1922; Payne, 1926; Pollister, 1930; Johnson, 1931 and others). The mitochondria in the young spermatid are composed of an outer chromophilic and an inner chromophobic part. They literally run together, giving rise to the nebenkern (Gatenby, 1917; Bowen, 1922) which, as the spermatid grows older, becomes differentiated into a series of ultramicroscopic lamellae that are separated by inter-lamellar spaces.

In preliminary observations on the living spermatid by means of the phase-contrast microscope, we were able to confirm much of the structure seen in the nebenkern of fixed and stained preparations. However, it is evident that the structure of the nebenkern seen in Figure 1B is difficult to correlate with that observed in the electron micrographs. Notwithstanding the fact that we have examined over 150 nebenkern in different stages of differentiation, we apparently have not seen the lamellar structure present in Figure 1B. That this is true is evidenced by the fact that the lamellae seen in electron micrographs measure only about 400 Å in width, a figure beyond the range of light microscope resolution. At present we are unable to explain this apparent discrepancy in structure of the nebenkern revealed by the light and electron microscopes; it may simply be that we have never observed under

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FIGURE 16. Stage in transformation of spermatid into spermatozoon. Note centriole at base of nucleus (N) and part of tail filament (T) between the elongating halves of the nebenkern. 2% osmic acid fixation. × 5000.

FIGURE 17. Basophilic body (C) cut through its long axis. Note electron-opaque lamellae or filaments and interlamellar spaces. The nucleus is shown at N. Buffered 2% osmic acid fixation. × 5000.

FIGURE 18. Body at C is of the same type as that in Figure 17C, but cut in cross section. The body at A is a developing nebenkern. Buffered 2% osmic acid fixation. × 6000.

the electron microscope a nebenkern possessing a structure of the type seen in Figure 1B.

The interesting part of this paper is, how does the ultramicroscopic structure of the nebenkern compare with that of other forms of mitochondrial material? It will be recalled that Palade (1952, 1953) demonstrated within the mitochondria a system of internal ridges or cristae that protrude perpendicularly from the inside surface of the membrane toward the interior of the mitochondria. Sjöstrand (1953) further observed these membranes to be double and to extend across the mitochondria. Since the cristae appear in the mitochondria of so many different types of cells, Palade (1953) suggests that they constitute a characteristic pattern of mitochondrial ultrastructure. However, in the mitochondria of the male germ cells of *Helix* instead of the characteristic cristae, a series of longitudinally oriented lamellae of about 400 Å in width were observed (Beams and Tahmisian, 1954). The same mitochondria show, when examined under polarized light, a positive birefringence in their longitudinal axis. If the grasshopper nebenkern is constructed like an onion (that is, the lamellae corresponding to the layers), then it may be said that the ultrastructure of both *Helix* germ cell mitochondria and grasshopper nebenkern are similar. However, it is difficult to determine what relationship, if any, exists between the lamellar structure of the nebenkern and the cristae structure of certain other mitochondria. Accordingly, it would seem that the exact type of internal ultramicroscopic structure in mitochondria material may vary, but that some form of structure must be present to provide compartments or increased intramitochondrial surface area which in some way enhances their enzymatic function. It is not known what role the mitochondrial material may play in the metabolism of the grasshopper spermatid. However, if there exists a correlation between structure and function within the mitochondria, as seems evident, the nebenkern with its remarkable structure should constitute a rewarding source of material for enzymatic studies.

It has long been known that certain material in the cytoplasm of many different types of cells has a special affinity for basic dyes. Hence, these bodies whose significance has not been wholly understood have been termed basophilic bodies or ergastoplasm (Wilson, 1925; Monné, 1948). Recently the electron microscope has revealed in different types of cells a group of filaments that are thought to be equivalent to the basophilic material (Dalton *et al.*, 1950; Bernhard *et al.*, 1952; Beams *et al.*, 1952, and others). According to Porter (1953), the fibrous nature of the basophilic material, which he has termed endoplasmic reticulum, is in reality an artifact, and the real structure is a system of canaliculi and vesiculated strands. The bodies that we have termed basophilic here seem to possess a structure similar to that in other types of cells. However, it is difficult to determine whether or not the lamellae are composed of single membranes or of double membranes with an intervening space between them. We are inclined to accept the latter view. Certainly the diffuse form of the basophilic material like that in Figure 13, appears vesicular.

It will be noted that in Champy-fixed material a more fibrous type of cytoplasm is revealed than occurs following fixation in buffered osmic acid; yet the structure and distribution of the lamellae within the nebenkern are much the same following either type of fixation.

CONCLUSIONS

1. The nebenkern of grasshopper spermatids arise by the fusion of mitochondria.

2. The mature nebenkern is composed of concentric lamellae about 400 Å in thickness. Some appear to be composed of double membranes. The lamellae are arranged like the layers in an onion and they alternate with layers of matrix.

3. As the spermatid transforms into a spermatozoon the nebenkern loses its lamellar structure, divides, and its two halves elongate down the middle piece of the developing sperm.

4. Some spermatids contain diffuse vesiculated bodies (endoplasmic reticulum) which are believed to be basophilic material. Other spermatids show large bodies composed of lamellae which are thought to represent a collection into one body of the basophilic material.

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STUDIES ON THE UREASE OF THE EGGS AND EMBRYOS OF THE SEA URCHIN, STRONGYLOCENTROTUS PURPURATUS [1]

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From studies on the comparative physiology of animals, it has been found that the kind of nitrogen excretion products tend to be correlated with environmental conditions, the most important single factor being the availability of water. One might suppose, on the basis of environment, that if sea urchin eggs excrete nitrogen it would be excreted in the form of ammonia since ample water is available for the rapid removal of this substance from the eggs and developing embryos. There are, in fact, reports of ammonia excretion by sea urchin eggs. Örström (1941) demonstrated a marked but transitory excretion of ammonia by sea urchin eggs immediately after fertilization and provided some evidence that this is a result of the deamination of adenosine to inosine by adenosine deaminase. Hutchens *et al.* (1942) confirmed Örström's findings, but attributed the ammonia production to amino acid deamination. A number of workers have investigated nitrogen metabolism during later development with results which, though in some measure conflicting, suggest that the early development of the sea urchin egg is characterized by little if any nitrogen excretion. Hayes (1934) reported an increase in total nitrogen during the first four hours of the development of *Echinometra lucunter*, followed by a small decrease between the 6th and 24th hours of development. Ephrussi and Rapkine (1928) found a decrease in total nitrogen from 10.7% dry weight to 9.7% during the period of development between 0–40 hours of *Paracentrotus lividus*. Hayes' data are complicated by the possibility that variable amounts of the gelatinous coat of the eggs, a glycoprotein (Tyler, 1949a), may have been present in different stages, since no special account of this seems to have been taken in this work. Ephrussi and Rapkine's results, as indicated by Horowitz (1939), also show an increase of 5.6% in the dry weight of the embryos during the period of development mentioned which would virtually cancel out any nitrogen loss during this period. Horowitz (1939) presented data for the nitrogen content of the eggs of *Urechis caupo*, a marine echiuroid worm, indicating that the nitrogen content is constant up to 24 hours of development (feeding trochophore). Though this last mentioned work admittedly has no direct bearing on nitrogen excretion in the sea urchin egg, the environment of the two forms is similar and one might expect, therefore, a similarity in nitrogen excretion products. Finally, it is reported by Gustafson and Hasselberg (1951) that the nitrogen content of developing embryos of *Paracentrotus lividus* remains constant for the first 32 hours of development.

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The present investigation is concerned with the enzyme urease and metabolically related enzymes, and the possible relation between these enzymes and the low nitrogen excretion by the developing sea urchin embryo. Since urease is usually thought of as an "excretory" enzyme, its existence in an embryo which may not excrete nitrogen during the morphogenetic period leading to the larval stage becomes of some interest.

MATERIALS AND METHODS

Eggs and sperm of *Strongylocentrotus purpuratus* were collected after the animals had been induced to spawn with isotonic KCl (Tyler, 1949b). The gelatinous coat of the eggs was removed with acid (pH 3.5) sea water (Tyler, 1949a), and the eggs were used if they were 90–100% fertilizable. Embryos were grown in approximately 1% suspensions at 11° C. in slowly rotating Erlenmeyer flasks. At the desired stage of development, samples were taken from the suspension. Swimming stages were concentrated by means of a specially constructed Lucite centrifuge similar to the Foerst plankton centrifuge. The embryos were frozen and stored for not more than 24 hours before *in vitro* assay for urease activity.

For *in vitro* determinations, the frozen material was homogenized and diluted with sea water to give a final concentration of about 0.5 ml. of packed eggs per 2.0 ml. of brei. Two milliliters of this were transferred to a 10-ml. screw-cap vial with 4.0 ml. of 0.5 *M* phosphate buffer at pH 7.4. At zero time, 1.0 ml. of a urea solution containing 100 mg. of urea was added. The reaction was allowed to proceed for one hour at 29.5° C., when the vials were chilled and centrifuged for 2–3 minutes at 3° C. Five milliliters of the supernatant were transferred to tubes containing 1 gm. of Permutit (after Folin). These were mixed for one minute, then washed three times with ammonia-free water. The Permutit was transferred to a Kirk-type micro-Kjehldahl still and alkalinized by the addition of 3 ml. of 20% NaOH. The ammonia liberated was distilled and titrated by the biiodate method of Ballentine and Gregg (1947). Kjehldahl nitrogen was determined on aliquots of the homogenates by the digestion method of Boell (1945) and the distillation and titration method of Ballentine and Gregg (1947).

For *in vivo* determinations, 6-ml. aliquots containing about 0.5 ml. of eggs were placed in a 10-ml. screw-cap vial, one ml. of sea water containing 100 mg. of urea was added and the eggs incubated for one hour at 18° C. The suspensions were then centrifuged lightly at 3° C. for one minute and 5.0-ml. aliquots of the supernatant were transferred to the Permutit. The eggs were washed with sea water, allowed to cytolyze in the original volume of cold distilled water and another series of 5.0-ml. aliquots taken from the supernatant. The cytolysis procedure permitted measuring ammonia that was produced by the reaction but failed to diffuse out of the eggs. The *in vivo* urease activity is taken as the sum of the ammonia liberated to the sea water plus that bound in the eggs and is expressed as micrograms of ammonia nitrogen per aliquot per hour.

Endogenous controls were run in all experiments and the data to be presented have been corrected by the subtraction of the control values. The endogenous ammonia production in breis was generally about 0.0075 microgram ammonia nitrogen per microgram egg nitrogen per hour, while for whole eggs, the usual figure was close to 17 micrograms ammonia nitrogen per aliquot per hour for unfertilized eggs and 20 for fertilized, varying slightly with the number of eggs per aliquot present in different experiments.

RESULTS

To ascertain the *in vitro* saturation point of the enzyme with substrate, two experiments with equal aliquots of eggs and varying urea concentrations were performed. The amount of urea per vial ranged from 2.5 to 20 mg. in one experiment, and from 10 to 90 mg. in the second experiment. The results indicate complete saturation with 30–50 mg. of urea but, as a safety factor, 100 mg. of urea per reaction vial were used in all *in vitro* determinations of urease activity.

Since urease in other organisms depends on reduced sulfhydryl groups for activity (Sumner and Poland, 1933), a check was made on the redox potential of the brei-buffer mixture using a Beckman pH meter equipped with a platinum electrode. The brei-buffer was found to have a redox potential of 150–170 mv. On the addition of one mg. of glutathione, this was lowered to 90–100 mv. The enzymatic activity was not appreciably changed in the presence of glutathione; values obtained were 0.0365 microgram ammonia nitrogen per microgram egg nitrogen per hour without glutathione and 0.0380 for the vial containing glutathione. As will be seen later, these results do not differ significantly from each other. In view of the failure of glutathione to increase the activity of the enzyme, and in view of the optimum redox potential of 100–200 mv. reported by Sizer and Tytell (1941) for jack bean urease, which is within the range obtained for the brei-buffer mixture, glutathione was not included in the subsequent assays.

To determine the reproducibility of the homogenizing, sampling and assay methods employed, embryos were raised to the hatching blastula stage and harvested. Three aliquots of these embryos were homogenized separately and their urease activities determined. Activities of 0.0361, 0.0368, and 0.0328 unit (microgram ammonia nitrogen/microgram egg nitrogen hour) were obtained, from which it is concluded that the determinations are reliable to about 0.005 unit.

Using the conditions for assay established by the above experiments, determinations of the activity of the enzyme were made on breis of fertilized and unfertilized eggs and on various stages during the first 114 hours of development. These results are summarized in Figure 1. It is seen that while there is little change in the activity of breis between fertilized and unfertilized eggs, there is a drop of about 30% by the unhatched blastula stage, and that this level is maintained to the pluteus stage, at which time the animals are beginning to feed. Adult tissues were analyzed in order to see if any further change in the activity of the enzyme occurred. Ovary, from which the eggs had been removed by KCl-induced spawning, and gut were assayed. Activities of 0.0285 and 0.0585 unit, respectively, were found. It would seem that the activity of urease in the adult animal is maintained at approximately the level found during embryonic development.

That urease activity *in vitro* does not change when the eggs are fertilized is indicated above. There still exists the possibility of an *in vivo* change. To test this, equal aliquots of eggs were assayed in the manner described above. For two separate experiments, the results are: experiment 1, 126.8 micrograms ammonia nitrogen per hour per aliquot unfertilized and 99.9 fertilized; experiment 2, 127 unfertilized and 119 fertilized. These experiments show little or no change in *in vivo* urease

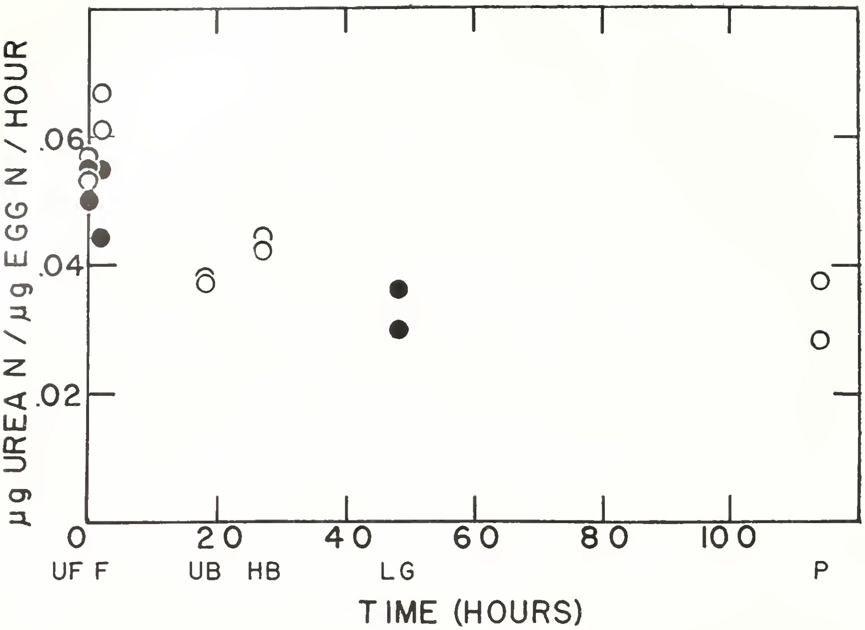

FIGURE 1. The *in vitro* activity of urease of *Strongylocentrotus purpuratus* as a function of time and developmental stage. UF, unfertilized; F, fertilized; HB, hatched blastula; LG, late gastrula; P, pluteus.

activity accompanying fertilization; the differences noted are certainly not comparable to the 4- to 6-fold increase found in respiratory rate on fertilization in sea urchins.

Experiments were done to determine if a change in urease activity resulted from homogenization. Equal aliquots of a suspension of unfertilized eggs were taken; one series was homogenized and the other was left intact. The temperature was 18° C. and the pH was 6.8 for the homogenized samples and 7.8 for the sea water in which the intact eggs were suspended. Though there is an apparent difference in the pH of the two samples, the enzyme may be considered to be acting at the same pH in both cases since the pH of the interior of the egg may be taken to be about 6.8 (Pandit and Chambers, 1932). The intact eggs produced 70 micrograms of ammonia nitrogen per hour per aliquot while the homogenized eggs produced 238.

There are numerous possible explanations for this four-fold increase in activity. One of these, the possibility that the eggs are relatively impermeable to urea, was tested by assaying for unfertilized *in vivo* activity with increasing amounts of urea. If urea is freely permeable, the *in vitro* saturation point (30 mg.) should also be reached *in vivo*. Two such experiments were done employing urea concentrations from 10 to 100 mg. per vial. The results, plotted in Figure 2, indicate that the enzyme is not saturated at 100 mg. of urea per vial (0.24 *M*) and therefore that the amount of substrate that reaches the enzyme is limited, presumably by permeability, assuming that urea is not being utilized by the eggs during the experiment. It should be noted that eggs incubated with urea were fertilizable to the same extent as endogenous controls (75–80%) after one or two washings with fresh sea water.

In order to have a more complete picture of the role of urease, it would be helpful to know how urea might be formed in the eggs. There are several pathways of urea formation, of which two are the ornithine cycle and the degradation of purines. Since the presence of a system such as the ornithine cycle, which would manufacture urea at the expense of energy only to have it immediately broken down again, seemed unlikely, it was decided to test for various enzymes of purine degradation. Homogenates of unfertilized eggs were examined qualitatively for the presence of adenase, xanthine oxidase, uricase, allantoinase, and allantoicase. Adenase was not demonstrable in more than trace amounts in two independent experiments using the method employed for urease, but with adenine as substrate. Xanthine oxidase was tested for using a modified Thunberg technique with both xanthine and hypoxanthine as substrates. Both substrates gave decolorization times of 10–15 minutes at 29° C. and pH 7.4, while in the absence of substrates, decolorization did not occur within 40 minutes. It is concluded that the enzyme is present. Uricase activity was measured in a Warburg apparatus. Two ml. of brei (0.5 ml. packed eggs per 2 ml.), to

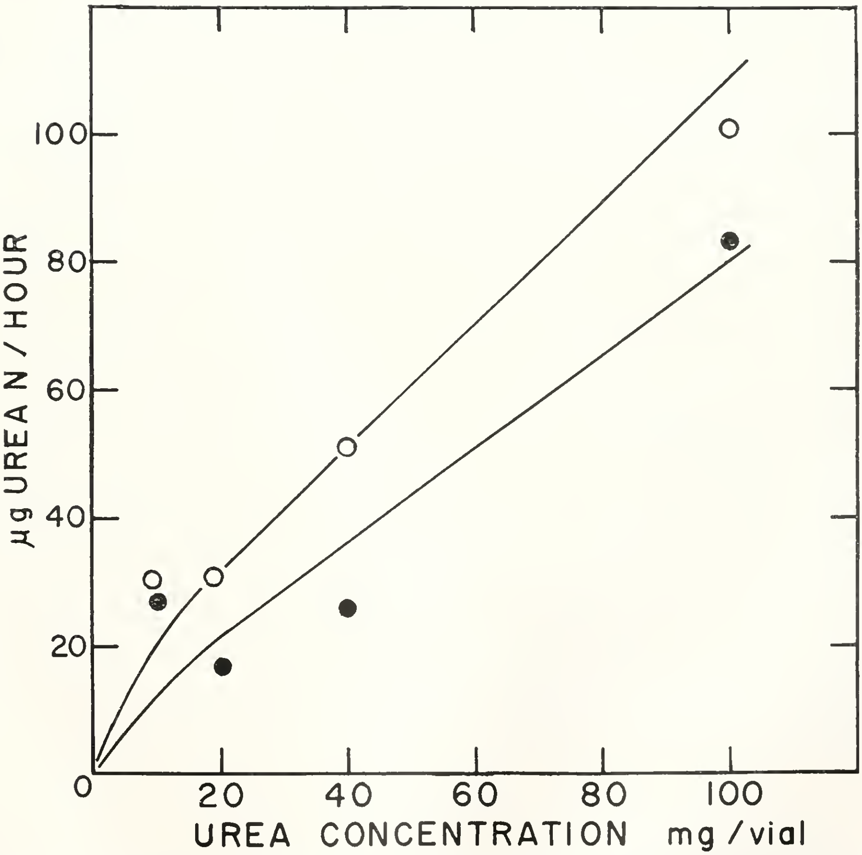

FIGURE 2. The *in vivo* activity of urease in unfertilized eggs of *Strongylocentrotus purpuratus* as a function of concentration of urea in the sea water.

which were added 20 mg. uric acid, gave an oxygen uptake at pH 9 and 30° C. of 10.0 and 14.0 microliters of O_2 per hour per flask in two separate experiments, whereas endogenous controls showed rates of 3.1 microliters of O_2 per hour per flask in both experiments; uricase is therefore present in the brei. Allantoinase and allantoicase were assayed simultaneously using the *in vitro* urease method, but with allantoin as substrate. Duplicate reaction vials contained 5.0 ml. McIlvaine's buffer at pH 7.4, 1.0 ml. undiluted egg homogenate, and 1.0 ml. allantoin solution containing 5 mg. of allantoin. In the duplicates 97.2 and 87.1 micrograms ammonia nitrogen were liberated in one hour at 22° C. These values have been corrected for a small endogenous ammonia production. It is concluded that allantoinase and allantoicase are present.

These experiments indicate that four of the enzymes metabolically related to urease are present in the unfertilized eggs. In addition, adenosine deaminase has been reported by Örström (1941) and Gustafson and Hasselberg (1951). It is probable, therefore, that the scheme of purine degradation typical of animal tissues exists in these eggs. With this conclusion, the function of urease in the sea urchin embryo might be expected to be that of completing the degradation of purines to ammonia, preparatory to its elimination from the organism as in a non-cleidoic system where water supply is not limiting. Against this, however, are the investigations cited earlier that indicate little or no nitrogen is lost by sea urchin embryos. The question of ammonia excretion in *Strongylocentrotus purpuratus* was examined directly by experiments in which the ammonia liberated by embryos into the sea water culturing medium was measured. One experiment covered the period from 20 minutes to 12 hours after fertilization, and a second was concerned with the interval from 24 to 60 hours after fertilization. In both, a 0.2% suspension of eggs was inseminated and, after washing, was cultured for the incubation period in fresh sea water at 11° C. and pH 8. Ammonia was determined on 5.0-ml. aliquots of the sea water both before and after the incubation period. No detectable ammonia was liberated by the larvae during the developmental periods tested.

With the establishment of the likelihood that nitrogen is excreted in very small quantities, if at all, during the early development of the sea urchin, it follows that urease is not primarily involved here with ammonotelic excretion. It is probable, then, that any ammonia produced by urease would be added to the metabolic pool of nitrogen to be used in the synthesis of various nitrogen compounds. Hultin (1950) has shown that the eggs of *Paracentrotus lividus* are able to incorporate N^{15}-labelled ammonia from sea water into proteins, a finding which tends to indicate the existence of such a metabolic pool. In this view, urease and the other enzymes of purine breakdown shown to be present are to be considered as factors in the mobilization of nitrogen for the many syntheses of the sea urchin embryos. An interesting possible source of purine for their action may be ribonucleic acid, with which these eggs are richly supplied.

SUMMARY

1. The urease activity of sea urchin eggs and embryos has been followed *in vitro* to the pluteus stage. The activity was found to decline by about 30% upon reaching the blastula stage, after which it remains relatively constant.

2. Adult sea urchin tissues were found to contain urease of activity comparable to that of the eggs and embryos.

3. The *in vivo* activity of urease in fertilized and unfertilized eggs was compared, with little or no change on fertilization noted.

4. The *in vivo* activity was found to be considerably lower than *in vitro* activity when measured under the same conditions, and evidence indicates that the *in vivo* activity is limited, at least in part, by the rate of penetration of urea into the eggs.

5. Of the enzymes usually involved in the formation of urea from purines, xanthine oxidase, uricase, allantoinase and allantoicase are shown to be present in sea urchin eggs, whereas adenase was not demonstrable with the method employed.

6. No ammonia within the range detectable by the methods employed was liberated into sea water by embryos during the periods of 20 minutes to 12 hours after fertilization, and 24 hours to 60 hours after fertilization.

7. The results suggest that urease and the related enzymes function to add nitrogen from purines to the metabolic ammonia pool during the pre-feeding stages of development.

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CYTOLOGICAL AND CYTOCHEMICAL STUDIES OF OOGENESIS OF POPILIUS DISJUNCTUS ILLIGER (COLEOPTERA–POLYPHAGA)

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The cytological events encountered in studies of gametogenesis pose many problems of interest to the cytologist. Although studies of spermatogenesis and oogenesis are of equal importance from the biological viewpoint, apparently much less attention has been given to the latter since the early part of the present century.

As pointed out by Wilson (1928), among the more important aspects of oogenesis are such problems as the morphological and physiological relations of the developing oocyte to the so-called accessory cells associated with it, the mechanism of oocyte growth and the formation of the yolk substance. The present paper is concerned mainly with the early development of the oocyte and its relation to the accessory cells of the ovary. Studies on the phases of oocyte growth and yolk formation are in progress and the results will be published elsewhere.

Broadly speaking, insect ovaries may be classified into two main types depending upon the presence or absence of so-called nurse cells. Thus the panoistic type (nutritive cells are lacking) is characteristic of the Orthoptera, Odonata, Isoptera and Aphaniptera while the meroistic type is characteristic of Coleoptera, Neuroptera, Hymenoptera and Hemiptera.

If the location of the nurse cells is taken into consideration, then the meroistic type may further be subdivided into the polytrophic condition, where nutritive or nurse cells alternate with the oocytes, and the telotrophic condition, in which case the nurse cells are confined to the apices of the ovarioles.

There is, however, some confusion in the literature with respect to the correct placing of families, especially in the case of the Coleoptera. Thus Imms (1948), following the classification of Stein (1847), considers the sub-order Polyphaga to be characterized by the telotrophic condition and the polytrophic condition to be characteristic of the sub-order Adephaga. On the other hand, Wigglesworth (1939) reverses the classification of Imms and Stein and in this respect appears to be following Weber (1933). Finally, Deegener (1928) cites the Coleoptera-Polyphaga as possessing the telotrophic type of ovary.

Hence, according to Imms, *Popilius disjunctus* should possess ovaries of the telotrophic type, while according to Weber and Wigglesworth they should possess polytrophic ovaries. More recently, the structure of the ovariole was investigated by Krause (1946) who concluded that the ovarioles were of the typical telotrophic type. The cytological observations reported below support the conclusion that the ovarioles of *Popilius disjunctus* exhibit a modified telotrophic condition.

The relationship between nurse cells and oocytes has been investigated by many workers; see for instance the papers of Giardina, Korschelt, Nussbaum-Hilarowicz

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(reviewed in detail by Wilson, 1928), Shaffer (1920) and others. Such investigations suggested that nurse cells were abortive ova specialized for the formation of nutritive materials to supply the needs of the developing egg. This problem was re-investigated in the Hemiptera by Schrader and Leuchtenberger (1952) using cytochemical procedures. Their findings showed that the nurse cells did contribute material to the egg cytoplasm and furthermore that this nutritive substance was in part derived from the desoxyribose nucleic acid (DNA) of the nurse cell nuclei.

MATERIALS AND METHODS

Specimens of *Popilius disjunctus* Illiger (Passalidae) were obtained from a biological supply house as required. In addition a number of *Macrodactylus subspinosus* Fabricius (Scarabaeidae) were collected in Newton, Massachusetts, and used to supplement the studies on *Popilius disjunctus* material. Both families are members of the superfamily Lamellicornia.

A. *Fixation*

The gonads were dissected free of tracheoles and fixed in Carnoy's solution (3:1) for two hours. The tissues were washed several times in absolute alcohol, cleared in benzene and embedded in 56–58° C. Tissuemat. Sections were cut at 10 μ and at 5 μ in order to provide sections containing whole nuclei and thin sections of nuclei for subjection to various cytochemical procedures. It should be pointed out that the dissections were performed immediately or within three days of receipt of each shipment.

B. *Staining*

1. *Nucleic acids*

a. The Feulgen reaction. The Feulgen reagent was prepared according to the method of Stowell (1945) and sections were stained for two hours at room temperature following optimal hydrolysis (12 minutes) in 1 N HCl at 60° C. All slides were stained in the same batch of reagent though at different times.

b. Methyl green staining. The dye was first purified by shaking a 2% aqueous solution with chloroform until the washings became colorless. A calculated aliquot of the purified dye solution was added to the phenol-glycerin mixture as used by Pollister and Leuchtenberger (1949) to give a final dye concentration of 0.2%. It has been shown by Pollister and Leuchtenberger (1949) and by Alfert (1952) that pretreatment of the slides with hot reagents causes a lowering of the amount of methyl green bound by nuclei. Therefore the staining reaction was carried out at 37° C. for 1½ hours. Slides were then rinsed in ice-cold water, blotted and rinsed in tertiary butyl alcohol and differentiated overnight in fresh tertiary butyl alcohol. This procedure was carried out in order to insure chemical staining (Michaelis, 1947).

c. Azure B. Sections were stained in 0.2% watery solution of azure B at pH 4.0 for 3 hours at 40° C., according to the method of Flax and Himes (1952). After rinsing, the slides were differentiated in the same manner as for methyl green.

Sections were also stained by the methyl green and azure procedures following treatment with ribonuclease (see below).

d. Toluidine Blue O. Preliminary investigations of basic staining were carried out by staining sections for 15 minutes in a 0.25% aqueous solution of toluidine blue followed by a short differentiation in absolute alcohol.

2. *Proteins*

a. Millon reaction. The reagents were prepared and used as described by Pollister (1950); see also Bryan (1951).

b. Fast green. A 0.1% solution of fast green was prepared and used as described by Schrader and Leuchtenberger (1950) and by Bryan (1951).

3. *Additional techniques*

a. Ribonuclease. Ribonuclease was obtained from the Worthington Biochemical Sales Company. The enzyme was boiled for five minutes in a few drops of saturated ammonium sulphate solution in order to remove any proteolytic impurities. The resulting solution was then diluted with redistilled water to give a final enzyme concentration of 0.2 mgm. per ml. Slides were exposed to the enzyme solution for four hours at 45° C. Control slides were immersed in ammonium sulphate solution (0.2 mgm. per ml.) but at the same temperature and for the same amount of time as the test slides.

b. Periodic acid-Schiff reaction (P.A.S.). The reagents were prepared according to McManus (1948). Sections were oxidized with periodic acid (0.5%) for 5 minutes, rinsed in distilled water and stained in leucobasic fuchsin solution for 15 minutes. After staining in the leucobasic fuchsin solution the slides were passed through a bleach bath (3 changes of 10 minutes each) consisting of 10 ml. of 1 N HCl, 10 ml. of a 10% $K_2S_2O_5$ solution and 180 ml. of distilled water.

Following this treatment the slides were washed in water, dehydrated and mounted. In certain cases methyl green was used as a counterstain.

4. *Photometry*

Measurements of the amount of dye bound or the color produced in the tissue sections were made with an apparatus essentially the same as that described by Pollister and Ris (1947) and by Bryan (1951). However, instead of a Weston micro-ammeter, a General Electric micro-ammeter having a sensitivity of 0.0014 micro-ampere per millimeter was used to measure the photomultiplier output. By means of a calibrated Ayrton shunt (total resistance 10,000 ohms) the sensitivity level of the meter was adjusted to the optimal range for recording the output of the photomultiplier tube. Interference filters were used to isolate from the spectrum of an AH_4 mercury lamp the spectral lines used in making photometric measurements. Each line isolated was close to the absorption maximum of the color reaction concerned.

It was necessary to compare data obtained from slides prepared at different times. Therefore, in order to check on the reproducibility of the reactions used, sections from one ovary were mounted on each slide and used as a standard. The extinction value of the blank prepared for the Feulgen reaction was found to be negligible.

5. *Computation*

The transmittance data were obtained by the "plug" method described by Ris and Mirsky (1949) and by Swift (1950). In addition, whenever warranted, the mean optical path (MOP) of each nucleus was determined and used in the computations as described by Bryan (1951). The transmittance data were converted into extinction values ($\log_{10} 1/T$) and the extinction data were computed as described by Bryan (1951).

It is self-evident that the amounts of substance computed from extinction data are expressed in arbitrary units which are, however, related to the absolute amounts of absorbing substance present.

RESULTS

A. *Cytological Studies*

Since there is in the literature some confusion with respect to the classification of the Coleoptera, based on the structure of the ovariole, the cytological findings are reported in some detail.

In *Popilius disjunctus* the typical number of ovarioles is two per ovary while in *Macrodactylus subspinosus* it is six. The histological structure is essentially similar in both species. Unless otherwise indicated, the following observations are based on studies of *Popilius* material.

Histologically speaking, each ovariole may be divided into two major regions—the germarium and the vitellarium. A longitudinal section of an ovariole is depicted in Figure 1.

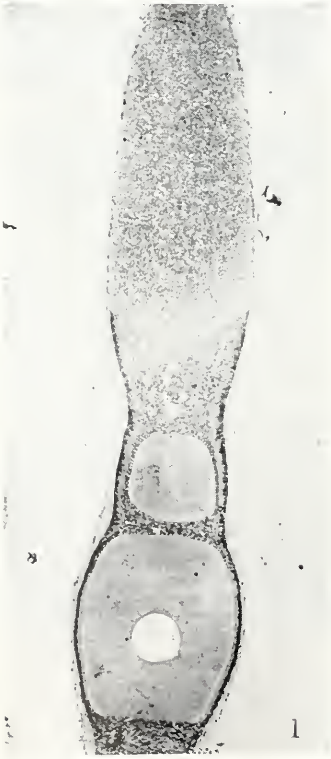
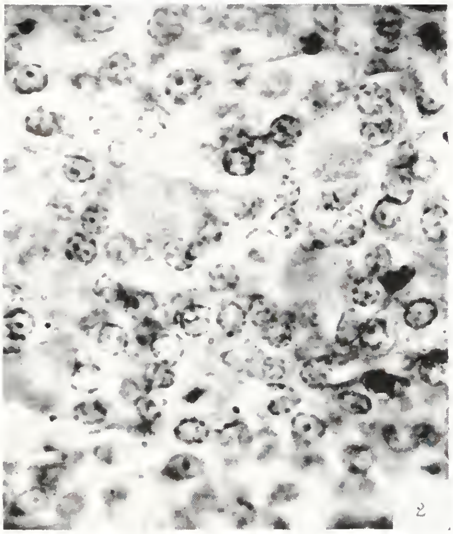


FIGURE 1. Longitudinal section through an ovariole from a mature adult. Stained with azure B. Photo 100 ×.

The germarium

At the apex of each ovariole the germarium becomes drawn out into an attenuated filament in the form of a spiral. As may be seen from Figure 2, this region in the immature female presents a distinctly different appearance from the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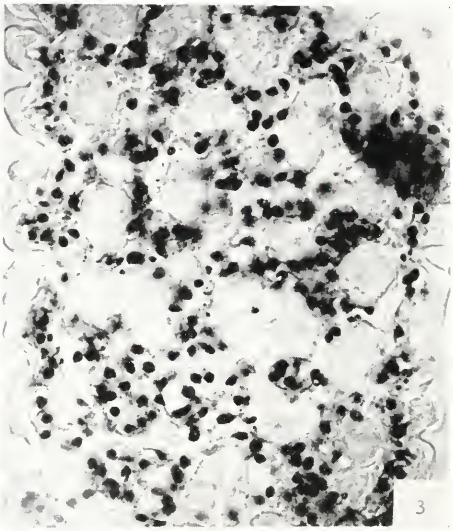
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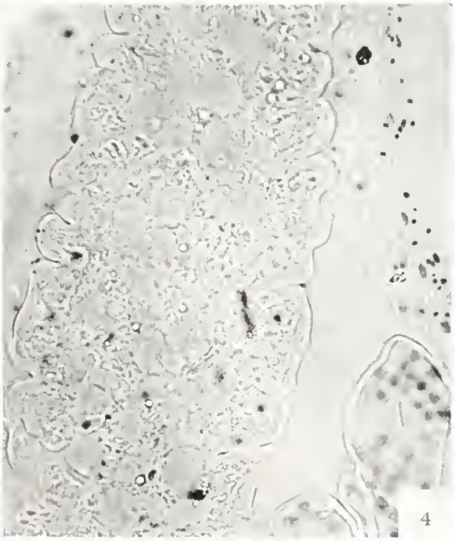
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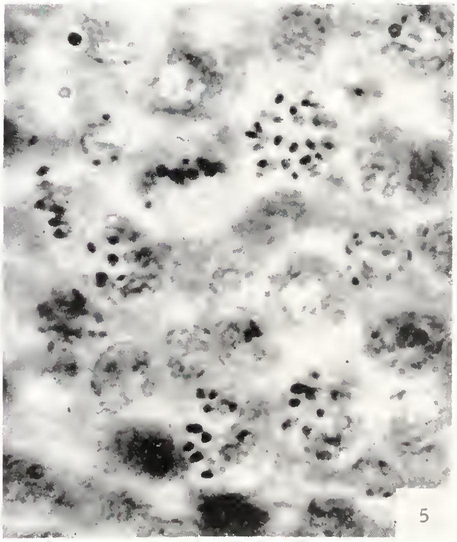
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FIGURE 2. Spiral tip of germarium of an immature adult. Note the masses of acidophilic material. Feulgen counter-stained with fast green. Photo 1200 ×.

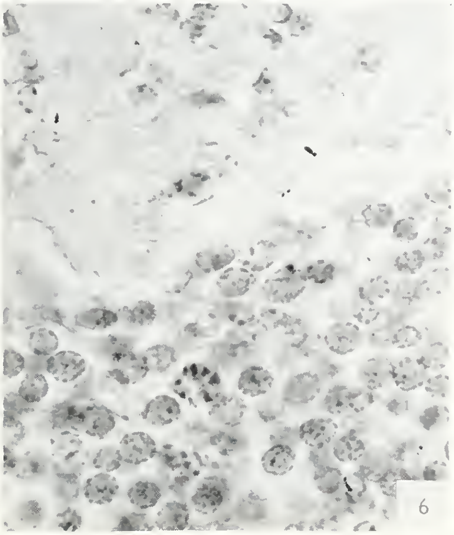
FIGURE 3. Similar region of germarium but from an individual a few weeks older than in the case of Figure 2. Stained with azure B. Photo 600 ×.

FIGURE 4. Spiral tip of germarium from a breeding female. Feulgen stain. Photo 400 ×.

FIGURE 5. Oogonial mitoses in germarium of immature adult. Feulgen counter-stained with fast green. Photo 3000 ×.

rest of the germarium. The cells appear to surround large masses of material which stains strongly with acid dyes as well as giving a positive Schiff (P.A.S.) reaction.

In older individuals the nuclei are slightly smaller but the chromatin is much more homogeneous. This condition is depicted in Figure 3. The apical region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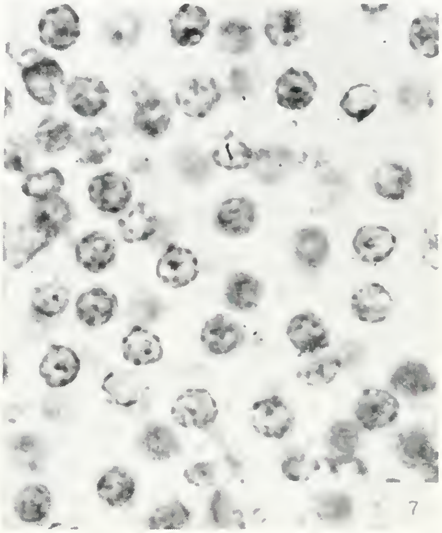
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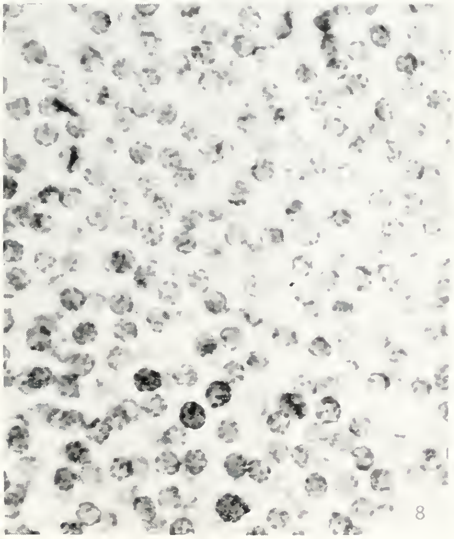
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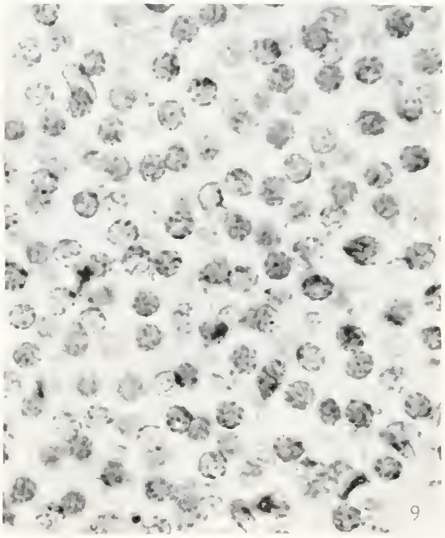
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FIGURE 6. The junction of the spiral tip with the body of the germarium. Feulgen. 1200 ×.

FIGURE 7. Nuclei from the body of the germarium of a young adult, showing partially condensed chromosomes. Feulgen stain. Photo 1700 ×.

FIGURE 8. Distal portion of germarium of a young adult. Feulgen stain. Photo 1000 ×.

FIGURE 9. Proximal portion of germarium from same ovariole as in the preceding figure. Feulgen stain. Photo 1000 ×.

of an ovariole taken from a laying female is illustrated in Figure 4. It is readily seen that the apical region is now almost devoid of nuclei. There is no evidence that this condition is brought about by pycnotic degeneration of the nuclei.

In the body of the germarium the cells are small and present a closely packed appearance; the nuclei are spherical and there is but little cytoplasm. Mitotic figures are abundant in this region of the ovariole of sexually immature females (Fig. 5). Counts of oogonial metaphase plates indicate the diploid number of chromosomes to be twenty-six, as previously reported by Shaffer (1917) and by Smith (1953). On the other hand, examination of ovarioles of mature females indicated mitotic figures to be absent from the germarium.

Close to the spiral tip, most of the nuclei exhibit a typical interphase condition (Fig. 6); however, nucleoli are extremely small or absent. More posteriorly, in young females, most nuclei appear to have been arrested in late prophase, the chromosomes being partially condensed and peripherally located (Fig. 7). Here, in sections stained by the Feulgen technique followed by counterstaining with fast green, there may be seen small globules or, occasionally, thread-like bodies which stain with the acid dye. This acidophilic material is usually located in the central region of the nucleus.

While the nuclei at any one level in the germarium show a uniformity of size, those in the proximal part show an increase in size such that the average nuclear volume is twice that of the younger (more distal) nuclei—compare Figures 8 and 9.

In the sexually mature female, the oocytes adnate to the vitellarium appear to be undergoing meiosis (Fig. 10). There is no indication that development progresses beyond the diplotene-diakinesis stage. Instead, the chromosomes become diffuse and cannot be identified clearly. It is at this time that the oocyte enters the growth phase.

The vitellarium

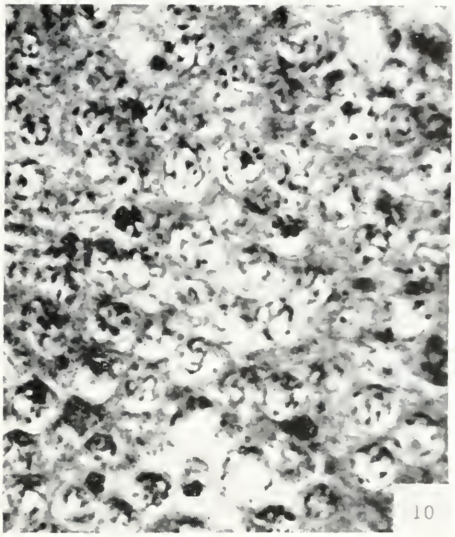
The zone of transition between the germarium and the vitellarium is depicted in Figure 11. Here the enlarging oocytes appear to be embedded in layers of cells greatly elongated in a direction perpendicular to the long axis of the ovariole.

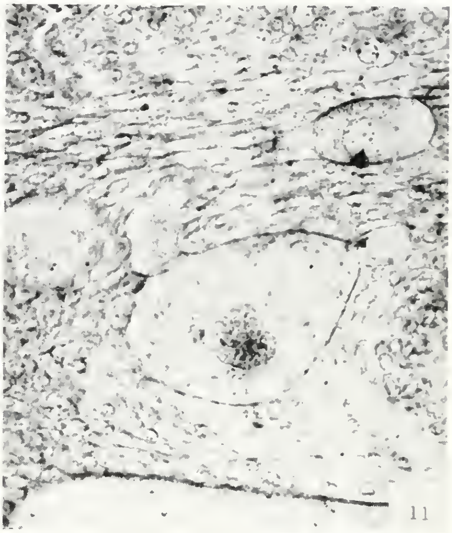
As the oocytes pass through this region they often develop cytoplasmic finger-like projections (Fig. 11). These processes cannot be traced beyond the distal border of the transitional zone. It would appear that they may be produced in response to the squeezing effect of the surrounding cells on the oocyte cytoplasm as the egg cells move through this zone into the body of the vitellarium.

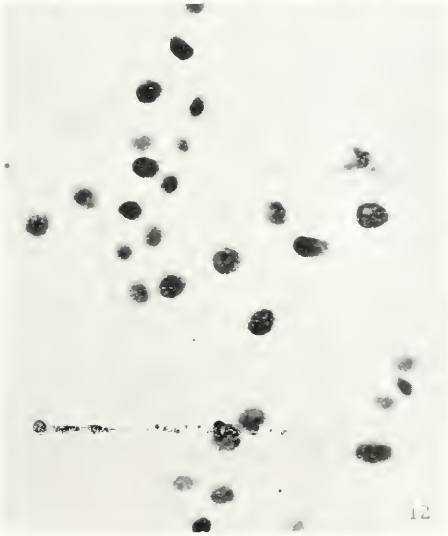
In the body of the vitellarium each oocyte can be seen to have acquired a sheath of follicle cells. In the case of the younger oocytes, the follicle is several cell layers thick whilst in older oocytes it has been reduced to a single layer of cells.

As the oocyte enters the growth phase there is a marked increase in volume of nucleus and cytoplasm. As the nucleus increases in size, there is a corresponding decrease in staining intensity; the mature nucleus is Feulgen-negative and does not stain with basic dyes. There is no evidence of marked nucleolar activity during the oocyte growth phase. If, however, protein stains are used, then it can be seen that the nucleus stains darkly at all times. As can be seen from Figure 11 the nucleus at first has a granular appearance. In older eggs, the protein stain is distributed quite homogeneously throughout the nucleus.

Cytological examination of the germarium (including the spiral tip) failed to reveal any signs of extensive cellular degeneration. No indications were found of the existence of plasmatic strands connecting the germarium with the developing eggs. In this respect the ovaries of *Popilius* differ markedly from the condition







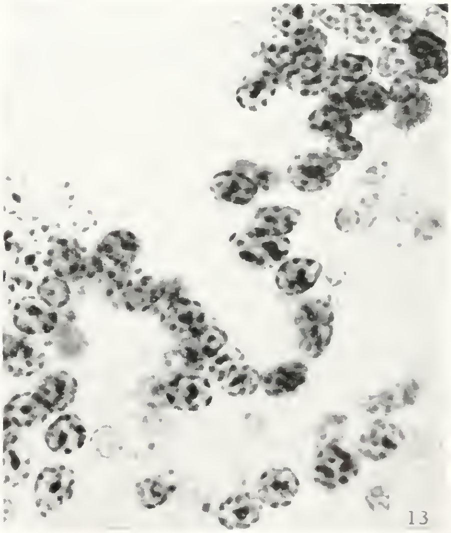


FIGURE 10. Portion of germarium adnate to the vitellarium, showing meiotic figures. Feulgen counter-stained with fast green. Photo 1500 ×.

FIGURE 11. The zone of transition between the germarium and vitellarium, showing an oocyte with cytoplasmic processes. Stained with the bromphenol blue-mercury reagent. Photo 500 ×.

FIGURE 12. Epithelium of the lateral oviduct, showing polyploid nuclei; compare with Figure 13. Feulgen stain. Photo 1200 ×.

FIGURE 13. Oviduct epithelium from the same section as figure 12. Note the clumps of heterochromatin. Feulgen stain. Photo 1200 ×.

found in the Hemiptera. Subsequent examination of the *Macrodactylus* material agrees with the observation made on the *Popilius* material.

In the vitellarium the eggs are arranged in a single row. Posterior to the oldest egg the ovariole is closed by a plug of epithelial cells which is ruptured at the commencement of oviposition.

Eggs from each pair of ovarioles are released into the lateral oviducts which unite to form the duct leading to the exterior. The nuclei of the cells lining these ducts exhibit several interesting phenomena. Here nuclear volumes fall into several classes simulating a polyploid series. While the smaller nuclei tend to be homogeneous (Fig. 12), the larger nuclei have a markedly different appearance (Fig. 13). Many strands of chromatin may be seen and, in most cases, they can be traced to clumps of heterochromatin located close to the inner surface of the nuclear membrane. Counts of these clumps, while somewhat variable, correspond closely to the diploid number of chromosomes. The amount of chromatin in each nucleus would appear to be several times as great as that found in the nuclei of other tissues.

It is obvious from the foregoing observations that the structure of the *Popilius* ovariole is quite different from that encountered in the Hemiptera and in other insects possessing the telotrophic type of ovariole.

B. *Cytochemical findings*

1. *DNA-Feulgen*

The data obtained are summarized in Table I. The values represent the mean extinctions, volumes and amounts of regenerated Feulgen dye per nucleus. In certain cases a few nuclei in the sample measured were small enough to warrant consideration of the MOP (see above) when computing amounts of DNA-Feulgen. Such values are, in the following table, indicated by a dagger (†) in the last column.

Measurements were also made on spermatid nuclei in order to provide data to be used as a standard, since the spermatid nucleus contains the haploid amount of DNA. These data are presented in Table I.

Cytological examination of the follicle cell nuclei showed the chromatin to be rather irregularly distributed. In addition, nuclei of follicle cells of older eggs are extremely large and show different degrees of flattening. For these reasons no photometric data pertaining to follicle cell nuclei are presented in this report.

2. *Methyl green*

In the present work it was repeatedly observed that the cytoplasm of oogonia, oocytes and follicle cells was stained by methyl green. In the case of the cells comprising the body of the germarium strong staining of the cytoplasm was observed; whereas the developing oocytes in the vitellarium, together with the investing follicle cells, evinced much weaker reactions. Overnight pretreatment of slides in the cold with dilute perchloric acid (Ogur and Rosen, 1950) abolished cytoplasmic staining. Complete suppression of cytoplasmic staining was achieved also by the use of the enzyme ribonuclease (see above).

TABLE I

DNA-Feulgen content of nuclei

Tissue and stage	Class	No. of nuclei measured	Mean extinction E_{549}	Mean nuclear volume microns^3	Mean amounts in arbitrary units
Testis-spermatid	I (n)	21	0.041	40.8	0.53±0.03*
Ovary: spiral tip of germarium					
(a) Immature adult	II (2n)	8	0.096	54	1.09±0.09
II→III	II *a*	24	0.132	60.5	1.60±0.02
	III (4n)	10	0.170	58.6	2.14±0.08
(b) Mature adult	II (2n)	16	0.190	43.1	1.12±0.03*
II→III	II *a*	13	0.143	44	1.46±0.03*
	III (4n)	2	0.162	43.1	2.05
Body of germarium					
(a) Immature adult Oogonia	II (2n)	19	0.086	70.4	1.11±0.04
II→III	II *a*	20	0.146	58.4	1.73±0.02
	III (4n)	11	0.191	64.6	2.42±0.08
(b) Mature adult oocytes—distal portion of germarium	III (4n)	20	0.188	58.4	2.27±0.09
III→IV	III *a*	2	0.232	68.5	3.13
	IV (8n)	3	0.350	52.8	4.96
Oocytes—proximal portion of germarium	II (2n)	3	0.090	52.8	1.35
III→IV	III (4n)	23	0.126	106	2.10±0.09
	III *a*	1	0.144	124	3.09
Epithelium of lateral oviduct	II (2n)	3	0.226	17.5	1.23
	III (4n)	15	0.269	30.4	2.06±0.13
	IV (8n)	27	0.461	39.9	4.31±0.14*
	V (16n)	29	0.641	72.5	8.86±0.18

* MOP considered.

3. *Azure B*

When used as described by Flax and Himes (1952), azure B stains DNA orthochromatically blue-green and RNA metachromatically purple. As is the case with other basic dyes, azure B failed to stain the nuclei of oocytes contained within the vitellarium. The pattern of cytoplasmic staining, obtained with azure B, is essentially the same as that obtained with methyl green. The staining of the cytoplasm was completely abolished by a prior digestion of the slides with ribonuclease.

4. *Protein methods*

It was found that the masses of acellular material in the spiral tip of the germarium stained quite strongly with fast green and less intensely with the Millon reagent.

In very young oocytes, before the growth phase has commenced, the threads of chromatin stain quite strongly while the cytoplasm is only weakly stained. In later oocytes, the nucleus presents a more homogeneous picture and, in contrast to the results obtained with basic dyes, still stains in an intense manner. In such nuclei the individual chromosomes can no longer be identified. As stated earlier, the nucleolar system does not show signs of intense or prolonged activity; only in a few older egg cells have small intranuclear bodies been observed. Studies on the protein content of oocytes are still going on and the results will be published at a later date.

5. *The periodic acid-Schiff reaction*

It was found that in the spiral tip of the germarium the acellular material which stained strongly with fast green also gave a strongly positive reaction with the Schiff reagent. In addition, small amounts of Schiff-positive material were distributed throughout the body of the germarium in an irregular fashion. In the cytoplasm of intermediate oocytes may be seen Schiff-positive droplets and granules. These structures are peripherally located, lying just below the surface of the egg. They are, therefore, in close proximity to the inner surface of the surrounding follicle cells. In much older eggs, where the chorion is present, the cytoplasmic Schiff-positive material is more uniformly distributed. It is also of interest that the tunica propria and the chorion give a strongly positive Schiff reaction.

Discussion

1. *DNA-Feulgen*

The germarium. From the data summarized in Table I it may be inferred that, in the immature female, the body of the germarium is essentially filled with oogonial cells multiplying rapidly by mitosis. The data indicate that the oogonial nuclei build up their DNA content to four times the haploid value prior to division—at which time the amount falls to that of the diploid value. Similar findings, in various animal tissues, have previously been reported by Swift (1950) and others.

The apical region of the germarium presents a similar picture of mitotic activity. Often strands of cells from the apical region appear to interdigitate with the most anterior layers of oogonial cells contained within the body of the germarium. The close correspondence of the photometric data, together with the cytological observations, suggests that the type of nucleus found in each region may be, in fact, identical.

In the case of the mature female the spiral tip, though mitotic figures were absent, still contains nuclei which possess an amount of DNA in excess of the diploid value. As reference to Table I shows, only two nuclei were found with the Class III amount of DNA; whereas Class II *a* (intermediate) and Class II nuclei were found in approximately equal numbers. The data show that mitotic activity continues in this region longer than in the body of the germarium. Perhaps the lack of Class III nuclei is an indication that mitosis has, by this time, slowed down or ceased.

According to Krause (1947), early in development a small group of primordial germ cells forms a knob-like structure at the distal tip of each arm of the Y-shaped gonad anlage. Later this knob gives rise to the spiral tip of the germarium. The central and basal portions of each anlage become hollowed out to form the cylindrical egg tube. This result is achieved by extensive cellular degeneration. Such degenerative changes were not reported to occur in the future spiral tip of the germarium.

Hence it may well be that most, if not all, of the functional primordial germ cells are separated from the rest of the aulage very early in development. Then it would follow that their derivatives, the definitive oogonia, as they are formed, pass into the body of the germarium where they multiply still further.

It is also possible that the primordial sex cells contained within the knob-like tip of the anlage are destined to become trophocytes or nurse cells. Such an interpretation would not be necessarily in conflict with the photometric data. It would be expected that such cells should undergo mitosis, during the time that oogonial divisions were taking place, in order to supply adequately the later nutritional needs of the oocytes.

On purely structural grounds, however, it would appear that such an interpretation should not be given undue emphasis for it is obvious that any nutritive material, in order to reach the growing egg cells, must diffuse through the entire body of the germarium.

At the present time it is not possible to discount fully the latter alternative. Though the presence of large amounts of PAS-positive substance in the apex of the germarium is suggestive of a trophic function for this region, it is clear that the apical zone cannot play as important a role in this respect, as does the end zone in the Hemiptera (see Schrader and Leuchtenberger, 1952). Such a conclusion is further supported by the absence of plasmatic strands connecting the apical region with the developing oocytes.

With respect to the DNA content, the condition prevailing in the body of the germarium of mature females is quite different. The data indicate that, with few exceptions, all the nuclei measured now have the Class III amount of DNA.

In the case of the proximal region three nuclei, out of a sample of twenty-seven, were found to possess the diploid amount of DNA. It is possible that these nuclei were the products of recent mitotic divisions and that the DNA content had not yet been built up to the final amount. It is also possible that these nuclei may be early follicle cell nuclei which were measured in error although this does not seem very probable for cytological reasons.

It was also observed that occasionally nuclei were encountered which were stained much more darkly than the typical oocyte nucleus, but which were of about the same size. In these observations perhaps lies the explanation for the finding, in the germarium, of nuclei possessing more than the tetraploid amount of DNA. It would appear likely that such nuclei, present in small numbers, are abnormal and do not give rise to mature eggs.

The vitellarium. The chromatin of the nuclei of oocytes in the zone of transition is distributed in an irregular manner. For this reason it was not possible to obtain valid photometric data.

It is well known that as the oocyte nucleus increases in volume, the intensity of the Feulgen stain and the degree of basophilia diminish. Nuclei of the developing oocytes in the vitellarium do not stain with basic dyes and also appear to be Feulgen-negative. Results such as these have often been interpreted to mean that there is no DNA in the nuclei of oocytes during the phase of growth. It is more probable that the decrease in intensity of the Feulgen reaction is attributable to a "diluting effect" of the increasing amounts of protein in the egg cell nucleus. This problem has been considered in more detail by Alfert (1950), and further remarks need not be made here.

As pointed out earlier, the follicle cell nuclei are not well suited to photometric needs. It is true that in the case of the younger follicles the nuclei are smaller and the chromatin is fairly homogeneously distributed. However, in these cases it was found that there was considerable overlapping of nuclei in the sections prepared. The degree of overlapping was such as to preclude the carrying out of photometric studies. It is of interest that, unlike the conditions obtaining in other insects, the cells of the more mature follicles do not appear to be multinucleate. Instead the volume of each nucleus has undergone a marked increase for it was found that the volumes of these nuclei were of the order of 1000 μ^3.

The lateral oviducts. Photometric measurements of DNA-Feulgen were made on nuclei of the epithelial lining of the lateral oviducts. The data are presented, in summary form, in Table I. It should be pointed out that only nuclei in which the chromatin was homogeneously distributed were chosen for measurement; no attempts were made to obtain data from nuclei such as those depicted in Figure 13.

It may be seen that the epithelial cell nuclei appear to contain amounts of DNA in multiples of the Class II (diploid) amount. It is also evident that nuclei containing the Class II amount are very infrequently met with, while nuclei possessing the Class IV or Class V amount of DNA constitute the most frequent classes.

The data suggest that there is a tendency for an increase in DNA content to be accompanied by an increase in nuclear volume. Whether or not a similar change in protein content occurs is unknown at the present time. Examination of the tissue involved failed to reveal any indication of metaphase plates or other phases of division. It is, therefore, quite probable that the mechanism underlying the formation of such nuclei, possessing amounts of DNA in multiples of the diploid amount, may be one of endomitosis. That the epithelial cell nuclei are endopolyploid in nature is further supported by the observations presented in the cytological section of this report.

This occurrence of polyploid cells in insect tissues is not unique for, as is well known, endopolyploid nuclei have been encountered in a variety of insects. Briefly, they have been reported by Geitler (1937) to occur in various tissues of *Gerris lateralis* and similar findings have been described by Berger (1938) with respect to the larval intestine of *Culex pipiens*. More recently, White (1951) has shown endopolyploid nuclei to be present in the testicular envelope of certain grasshoppers.

It is not possible at the present time to attach any functional significance to the present findings. Examination of the cytoplasm of these cells precluded the idea that the epithelial tissue may possess a glandular function. The existence of polyploid nuclei in the lateral oviduct tissue is perhaps a cytological indication of ageing.

2. *General considerations*

The basic dyes, azure B and toluidine blue, were used in conjunction with ribonuclease to study the cytoplasmic basophilia of oocytes and oogonia. During the course of these studies it was noted that the acellular material in the apex of the germarium stained, at best, very faintly with the dyes used. These observations indicate that the acellular material does not contain large amounts of RNA.

It would appear to be of some significance that the cytoplasmic basophilia of the follicle was found to be particularly intense during the periods of oocyte growth and chorion formation. It follows, therefore, that the follicle may be instrumental in supplying the growing egg with nutritive material. It would also appear that follicle cells are intimately concerned with the formation of the chorion.

It is a striking fact that in *Popilius*, nucleoli are never very prominent components of the nucleus. Perhaps the variable form of the nucleoli may be attributed to the absence of "effective" nucleolar organizing regions. Such a conclusion would not be in conflict with the observations since Heitz (1931) has reported the formation of nucleoli despite the fact that nucleolar regions were lacking.

That the nucleolar system, as envisaged by the Caspersson school, does not achieve prominence during the period in which oocytes are rapidly increasing in size may argue against the idea that, in *Popilius*, nucleoli play an important role in the growth process. At the present time, however, the mechanism of oocyte growth must remain an open question.

The life history of *Popilius* has been studied by Riley (1872), Wheeler (1923), Gray (1946) and others. Oviposition commences during May and continues until early July, the peak of activity being in June. According to Gray (1946), relatively few eggs are laid by any one female. The average number appears to be of the order of thirty-five and sixty appears to be the maximum.

From mid-August until late in October immature red-colored adults are found in the colonies; later than this, all individuals appear to be the black-colored mature adults. It is of interest that in the present work, no beetles taken from the colonies later than August proved to be the mature adults of the previous spring, indicating that death must occur shortly after the end of the reproductive phase.

Examination of the ovarioles from immature adults taken in late September revealed the presence of developing eggs in the vitellarium. Ovarioles of immature individuals taken a few weeks earlier proved to contain numerous oocytes undergoing meiosis. The stages found ranged from zygotene to diakinesis. It should be stated that the fixative used (Carnoy 3 : 1) does not preserve the meiotic figures well enough to permit identification of the stages with absolute certainty.

From the foregoing observations it would appear that the first eggs laid must have been present in the vitellarium for some months prior to the breeding season. However, this does not seem to be the case, for it was repeatedly observed that in individuals taken over the winter months, the oldest eggs appeared to be undergoing degeneration and resorption. In one case, the posterior part of the vitellarium had been invaded by phagocytes, suggesting that phagocytosis may be an important factor in the resorptive process. It would appear then that during the winter months mature females may actually rely upon the almost mature eggs and their follicles as a source of supplementary nutritive material. Individuals obtained a few weeks prior to the commencement of the breeding season failed to reveal any indication of degenerative processes in the vitellarium.

That unfavorable environmental conditions may be responsible for the degenerative process is perhaps further suggested by the following observations. Ovarioles taken from *breeding* females immediately upon receipt appear to be quite normal whereas indications of oocyte degeneration are apparent if such females are kept under laboratory conditions for longer than ten days. In addition, there are the observations of Gray (1946) to the effect that under laboratory conditions females do not lay for more than six or seven days and, furthermore, the last eggs to be laid under such conditions are usually abnormal in appearance.

SUMMARY

1. The cytology of the ovarioles of *Popilius*, together with their associated ducts, is described.

2. Cytological and cytochemical studies of the germarium suggest that the ovariole constitutes a modified telotrophic type.

3. Microspectrophotometric measurements of nuclei, stained by the Feulgen reaction, indicate that during the interphase period oogonial nuclei build up their DNA content to twice the diploid amount and that after division the daughter nuclei each contain the diploid amount. The data also indicate that in mature females primary oocyte nuclei contain the tetraploid amount of DNA.

4. Cytological observations, together with photometric measurements, suggest that the epithelium of the lateral oviducts is an endopolyploid tissue.

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WATER FILTRATION BY THE BAY SCALLOP, PECTEN IRRADIANS, AS OBSERVED WITH THE USE OF RADIOACTIVE PLANKTON[1]

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Studies of the movement of sea water through the mantle cavity by lamellibranch molluscs and the efficiency of the gills in removing particulate matter from this flow are of considerable interest to biologists concerned with investigations of the feeding activities of these bivalves. Only through a more complete understanding of the factors controlling the rate of water propulsion, the retention of particles by the gills, and the ingestion of filtered material can we approach problems in the nutritional physiology of these animals.

The rate of water propulsion of a number of species of lamellibranchs has been reported by investigators using either direct measurement techniques or indirect methods based on the reduction of the number of particles in a suspension in which the animals have been placed. Considerable work has been done on the factors affecting the rate of pumping of oysters using direct measurements of the rate of flow following, in general, the experimental arrangement described by Galtsoff, Chipman, Hasler and Engle (1938). The important part of this technique is the use of an "apron" developed by Nelson (1936). In many lamellibranchs direct measurements cannot be made and indirect methods have been widely used. About the first of these measurements that may be considered as truly quantitative was that of Fox, Sverdrup and Cunningham (1937). Their experiments were based on the chemical determination of changes in the calcium content of suspensions of calcium carbonate in which *Mytilus californianus* were placed. The use of plankton in suspension with photoelectric determination of changes in concentration resulting from the activity of the animals was suggested by Jørgensen (1943). Colloidal graphite was used by him in later studies (1949a, 1949b, 1952, 1953) in measurement of conditions affecting the pumping rate of filter-feeding invertebrates and the relation of this rate to metabolic activity. Rao (1953) used these techniques to advantage in his studies on the rate of water filtration of *Mytilus californianus* from different latitudes.

With the availability of methods of labeling plankton cells with radioactive isotopes and the extremely accurate technique of measuring changes in cell numbers based on the detection of small amounts of radioactivity contained in such cells, it seemed advantageous to employ these methods in studies of the filtration rates of filter-feeding invertebrates. Consequently, we have used plankton containing radioactive phosphorus in studies of the rate of water propulsion of the bay scallop, *Pecten irradians* Lamarck, one of the more active species of lamellibranchs, which could lead to further work on the factors concerned with filtration rate and feeding in these and other related marine molluscs.

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Materials and Methods

The scallops were collected from the vicinity of the Shellfish Laboratory of the U. S. Fish and Wildlife Service at Beaufort, North Carolina. They varied in size according to their age. They were all of the same year class, spawn of the fall of 1952, and consequently were rather uniform in size for any date of collecting. No very small scallops nor very large scallops were used. The smallest were those studied in June of 1953, and the largest those collected in October, 1953. Spawning of some scallops took place in the laboratory jars starting August 7 and was observed as late as early October. The biology of this species is well covered in a paper by Gutsell (1930).

Observations were made on single scallops immersed in suspensions of plankton cells in sea water. The volume of the suspension was usually 3 liters. With the small scallops a few observations were made in volumes of 1.5 liters, while the largest were tested in 6-liter volumes. The suspensions were well stirred by a stream of air bubbles. It was necessary to fasten the scallops to an L-shaped glass rod to prevent their swimming about. Since the scallop is very sensitive to the movement of an object in the near vicinity, the jars were well enclosed in a wrapping. When not under observation, the scallops were kept in running sea water.

The salinity of the sea water in the laboratory ranged from 31 to 36 parts per thousand, usually being about 34 parts per thousand. Heavy rains accompanying a hurricane in August reduced the salinity to a low of 14 parts per thousand, but no observations were made at the time of low salinity. The sea water used for preparation of the suspensions was aged sea water and plankton-free. It had a salinity of 35 parts per thousand. The scallops were not subjected to any significant change in salinity during the tests.

Observations were made at room temperature, which was held relatively constant throughout the summer and fall. The experimental suspensions ranged from 21.9° to 25.8° C. Variations during any one period of observation averaged 0.50° C., and only twice exceeded 1.0°.

Single species cultures of a diatom, *Nitzschia closterium* (56 microns in length), and a flagellate, a species of *Chlamydomonas* (7 microns in size), were made radioactive from the incorporation of radioactive phosphorus,[2] P^{32}, following the methods developed at our laboratory (Rice, 1953).

It is not difficult to culture many species of phytoplankton so that each cell is highly radioactive. These cells, when grown in enriched sea water containing virtually no phosphorus excepting the carrier-free radiophosphorus, will take up large amounts of the isotope. This isotope is a useful one emitting only β particles of rather high energy and very measurable. Since phosphorus is one of the major nutrients required, the P^{32} will be incorporated rapidly into organic compounds with little remaining in the exchangeable phosphorus of the cells. With rapidly dividing cells grown in this way, there is practically no radioactivity outside the cells in the sea-water medium.

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[2] Obtained from the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

The concentrated phytoplankton cultures, or portions of them, were added to the aged sea water to give the desired cell concentration. The number of cells present after dilution was calculated from measurements of aliquots of the concentrated culture using an improved Neubauer haemocytometer. Changes in the cell concentration of the suspension were followed by radioactivity measurements of aliquots removed at regular intervals. In some experiments samples were taken at various locations in the jar. No significant differences were noted, and the measurements made for each time interval were averaged. Control jars without scallops were sampled in order to measure the rate of settling of the suspensions. Although measurable, settling was so slight as to be negligible. Since the cell population after dilution could be calculated from exact microscope counting, and the radioactivity of the cells accurately measured, any changes in cell population could be detected even though extremely slight. It would be quite impossible to detect such slight changes occurring in short periods of time in dilute suspensions using electrophotometer measurements. In fact the original starting concentration was often lower than that which would give a measurable difference from ordinary sea water blanks using such equipment.

The radioactivity of 10-ml. aliquots of the plankton suspension was measured after the addition of nitric acid to disintegrate the cells and dilution to 25-ml. volumes. A dip type Geiger-Müller (G-M) counting tube was used immersed to a fixed depth in a 50-ml. centrifuge tube and connected to the conventional type scaler. Necessary correction for radioactive decay of the P^{32} was made.

It was originally thought that observations with the G-M tube immersed directly in the suspension would do. However, there was soon an accumulation of radioactive cells around the tube even though it was treated with the non-wetting agent, Desicote. Another objection was the limited time of measurement which might not provide enough counts to give the desired accuracy.

Dilution of the phytoplankton culture with sea water to give the experimental cell concentration was great and very little P^{32} was in the sea water of the suspension. Nearly all the radioactivity was within the cells. However, duplicate aliquots of the suspension were filtered through a freshly-prepared barium sulfate precipitate held on a No. 42 Whatman paper and the small amount of radioactivity of the filtrate averaged and subtracted as an additional background for each observed reading. The preparation of this very retentive filter was described by Rice (1953). Filtration at the end of the observations showed the radioactivity measured was due to the presence of cells.

Results

On being immersed in an experimental phytoplankton suspension, the scallops opened almost immediately and soon started to filter the water through their gills. This resulted in a lowering of the cell content of the suspension. A semi-logarithmic plot of the cell concentrations measured at frequent intervals throughout the period of observations was made to visualize these changes. Figure 1 shows examples of the rate of clearing of suspensions of *Chlamydomonas* and *Nitzschia* cells with different concentrations of cells at the start of the experiments. The rate of clearing varied, but followed the same pattern in nearly all of the tests.

There was a gradual increase in the rate of reduction for a time following the immersion of the scallop. Generally, this took place rather promptly, although in a few instances the decrease in cell number was delayed slightly. This gradual increase in rate of reduction of phytoplankton cells at the start of the observations would be expected since the experiments were started with immersion in the suspension. It was, of course, necessary for the scallop to adjust itself to the new conditions and resume its filtering activities. There was no delay from failure to open as might occur in tests using other lamellibranchs. Normally scallops do not remain closed for any long period.

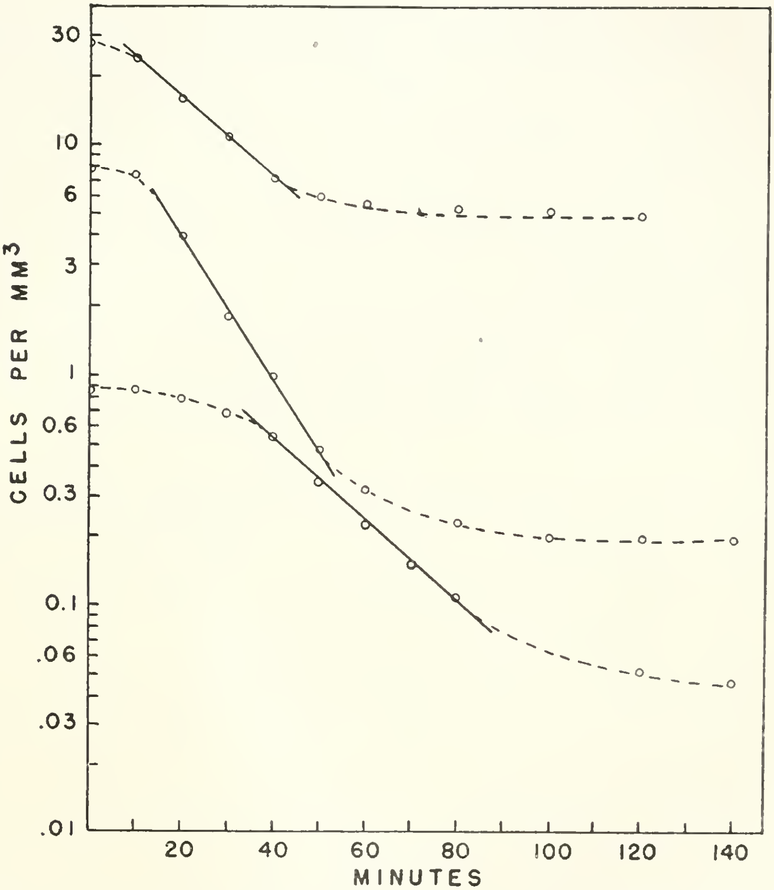

Figure 1. Changes in concentration of phytoplankton cells in suspensions with time after immersion of scallops. Upper curve was with use of *Chlamydomonas* cells, lower two with *Nitzschia*. The solid lines represent the calculated regression lines.

After the initial changes in the rate of particle reduction, a nearly constant percentage decrease of the suspended phytoplankton took place. The regression line for the logarithms of the cell concentrations observed during this decrease was calculated by the method of least squares and drawn for each of the experiments. The solid straight lines of the graphs in Figure 1 represent the calculated regression lines.

The observed points fall very close to these lines. In some instances it was possible to draw a straight line connecting the observed points.

If one assumes that the conditions of the experimental arrangement were satisfactory, the straight line decrease in the logarithm of the cell concentrations with time fits the mathematical equation for a constant rate of water filtration with complete removal of the suspended phytoplankton from the water filtered by the scallop. During this time of complete removal, the observed rate of decrease in cell concentration may represent the rate of water filtration of the scallop. Changes in the rate of clearing of the suspension can be interpreted as resulting from changes in the amount of water filtered. In our experiments it seems likely that the rate of clearing of the suspension was representative of the rate of water propulsion of the scallops during this period of the observations.

As the observations were continued, the decrease in phytoplankton became less and less. This resulted in a flattening of the curves. Although not shown in Figure 1, the number of cells in suspension gradually increased in instances where the observations were prolonged.

There are a number of factors concerned with determining the rate of reduction of particles in suspensions in which the scallops were immersed for observation of their filtering activities. Different ones of these may affect the rate at different times, or more than one factor may be acting at one time. The flattening of the curves of our observations, therefore, may not have resulted from a change in the rate of water filtration.

The decrease in rate of removal was apparently related to time in the suspension. It was not related to cell concentration. Series of observations were made starting the scallops in concentrations of either *Nitzschia* or *Chlamydomonas* at which there was a previous leveling-off in the rate of removal. In each series there was rapid removal followed by a less rapid rate, regardless of the starting cell population. This occurred even in starting concentrations of *Nitzschia* cells of 0.85 per mm.^{3} and in which a leveling-off of the curve took place at 0.04 cell per mm.^{3}.

Incomplete mixing of the filtered water with the unfiltered suspension would result in a decrease in the observed rate of phytoplankton removal that would get less and less as the observations were continued. Changes in the amount of stirring of the suspension in the jar were tried. Even rather violent stirring did not alter the shape of the curves in our experiments. A number of aliquots taken from various locations in the suspension did not appear to differ significantly from each other. Although it is realized that it is virtually impossible to get the mixing required to fully meet the requirement of the mathematical expression giving a straight line decrease, we believe that recirculation of filtered water through the scallop before mixing was not wholly responsible for the flattening of the curves we observed.

The decrease in the rate of removal reflected in the flattening of the curves could be interpreted as a change in the rate of water propulsion of the scallop. However, such a change was not apparent. The scallops were seen to be creating a considerable current of water even when there was no appreciable decrease in suspended plankton. This was also true when the cell concentration of the suspensions increased from a return of phytoplankton cells after earlier removal. It seems not unlikely that the change in the rate of clearing of the suspension was a result of a changed efficiency in the filtering by the scallop, together with a return of cells previously entrapped in the mucus of the gills.

Table I

Water filtration rates of scallops

Range in shell length (mm.)	Weight of meats (gm.)	Starting concentration (cells/mm.^{3})	Liters/hour filtered		Rate per scallop (l./hr.)	Rate per gram (l./hr.)
			Nitzschia suspensions	Chlamydomonas suspensions		
38–44		20		2.3		
		21	4.0			
		23	1.0			
		23		1.9		
		32		3.5		
		32	4.1			
		44	3.4			
		50		5.9		
		56		5.5		
		57		1.0		
Average	3.3		3.13	3.35	3.26	0.99
47–48		23	13.8			
		34	7.8			
		38		6.5		
		44		9.9		
		78	8.0			
		85	3.3			
Average	8.8		8.22	8.20	8.21	0.93
54–56		2.2	14.7			
		4.7	19.3			
		6.4		10.5		
		8.6		13.3		
		11	5.6			
		21	6.7			
		27		3.5		
		31		8.6		
		33		9.0		
		57	8.5			
Average	12.7		10.96	8.98	9.97	0.79
64–65		0.85	9.8			
		1.0	21.6			
		7.9	25.4			
		22	14.0			
		22	16.3			
		22	13.6			
		22	21.5			
		34		6.8		
		38	5.5			
		52	16.6			
		59	10.8			
Average	20.6		15.51		14.72	0.71

If one assumes that the scallop was filtering the suspension efficiently with complete removal of the suspended particles, it is possible to calculate the rate of water propulsion by the application of the formula given by Jørgensen (1943). This is

$$conc_t = conc_0 \cdot e^{-\frac{m}{M} \cdot t}, \text{ or } m = \frac{(\log conc_0 - \log conc_t) \cdot M}{\log e \cdot t}$$

in which M is the volume of suspension in liters, m is the quantity of water moved in liters per hour, while $conc_0$ and $conc_t$ are the cell concentrations at the initial time and after t hours of time. Undoubtedly the rates obtained are not absolute. They can only represent an approximation since the original assumption made is not likely to be entirely correct. Escape of the plankton through the gills, if occurring as a fixed percentage of the concentration in the suspension, would not change the nature of the curve but would alter the slope. The measured pumping rate may actually be lower than the true rate.

In our observations we can well assume that the changes in cell concentration in the suspensions represent efficient filtration with complete removal of the suspended cells by the gills excepting for the latter part of the observation times when flattening of the curves took place. We have therefore calculated the rate of water propulsion for the scallops for that time after adjustment to the immersion during which a semilogarithmic decrease in suspended phytoplankton cells occurred. The rates for observations of different sized scallops immersed in suspensions of *Nitzschia* or *Chlamydomonas* at the various concentrations tested are shown in Table I.

Individual scallops in observations made at different times differed in their rate of filtration, but under like conditions of test the rates were reasonably uniform. The average rates were the same for experiments using either species of phytoplankton for the suspended material. They did not appear to be related to concentration. As the scallops increased in size, their rate of water filtration increased. In June and early July the small scallops averaged about 3 liters per hour. Later during the summer they filtered increasing amounts, and by fall were pumping an average of nearly 15 liters per hour. The maximum rate observed was 25.4 liters per hour for a scallop measuring 65 mm. in length. We did not determine the rate of filtration of the scallops in late fall or early winter when the average size of this year class was somewhat greater.

Although the number of observations was small, the average rate of water filtration per gram of tissue was greater for the smaller scallops than it was for the larger. It is not known what effect the development of the gonads in the late summer may have had on the rate of filtration.

DISCUSSION

The use of radioactive plankton cells for determination of changes in the concentration of cells in suspensions in which a filter-feeding animal is placed is valuable for studies of the filtering activities of these animals. Since the cells can be made very radioactive, it is possible to use very dilute suspensions. It eliminates the need for growing large quantities of plankton in culture for such experiments. The suspensions can be made more comparable to normal sea-water environments. Even in a large volume of suspension in relation to the animal under observation, repeated frequent sampling with the withdrawal of small aliquots is possible. The accuracy of determining radioactivity in extremely minute amounts allows very accurate estimation of cell numbers. No attempts were made in the present study to explore the limits of determination of changes in cell concentrations, nor how few cells in suspension could be measured. However, the radioactivity of the plankton cells used could have been increased considerably, and larger aliquots could have been taken. In all the experiments the aliquots were diluted for radioactive counting and a counter with a rather thick wall (30 mg./cm.^{2}) was used.

The rates of water filtration were measured in cell concentrations well below those that were found by Loosanoff and Engle (1947) to interfere with the pumping activities of oysters. The concentrations used were within those that the scallop might encounter in nature. A short review of the literature on the estimation of phytoplankton abundance in the sea is given by Bainbridge (1953). Galtsoff (1949) presents a discussion of blooming of plankton in the sea. He reports the number of *Gymnodinium* in the surface water in a red tide off Florida as reaching a concentration of 56 cells per mm.^{3}. Cole (1939) measured the phytoplankton content of the sea water pumped into his experimental oyster tanks, and Gaarder and Spärck (1932), Alvik (1934), and Gaarder (1938) report observations for Norwegian oyster pools and fjords. Their values range from 4 to 24 cells per mm.^{3}. Recently, in Great Pond, Massachusetts, a tidal estuary, Hulburt (personal communication) observed a range of phytoplankton from 1.8 to 24 cells per mm.^{3} in carefully counted samples through different seasons. For more open waters, Riley (1941) states that the range he found on Georges Bank from September, 1939 to June, 1940 was from 0.15 to 85 phytoplankton cells per mm.^{3}. Except for most unusual situations, diatoms generally are not as abundant as flagellates and smaller nannoplankton. The importance of nannoplankton in the food-chain cycles of the sea has now become recognized. It is likely that the normal population of diatoms in the sea is about 0.5 cell per mm.^{3} (Hardy and Gunther, 1935). It can be seen that the cell concentrations used in our experiments may not be excessively high, with the possible exception of some in which the diatom *Nitzschia* was employed.

Although the indirect method of measuring the rate of water filtration of lamellibranch molluscs has been used in a number of studies, only in two papers has detailed information as to the changes in the rate of particle reduction during the time of observation been given. Fox, Sverdrup and Cunningham (1937) show data indicating a semi-logarithmic decrease in suspended calcium carbonate with time of observation in experiments using the California mussel. Such a decrease indicates complete removal of the suspended material from the water filtered by the mussels and a rate of filtration relatively constant for the time of measurement. They did observe, however, in certain preliminary experiments a gradual decrease in the rate of reduction with time. This did not take place in later experiments when adequate stirring was provided to minimize the effect of recirculation of filtered water through the mussels.

Jørgensen (1949a) compared the rate of water filtration of the mussel, *Mytilus edulis,* when immersed in suspensions prepared with colloidal graphite, the diatom *Nitzschia,* and three species of flagellates. Changes in the concentration of the suspensions were determined photometrically. The rates of filtration, calculated on the reduction of suspended material, varied with the type of particle used and often changed during the period of observation. In his experiments using graphite particles, there was a gradual decrease in the rate of clearing; the rate at the beginning of the experiments was greater than at the end. In a few observations in which the phytoplankton constituted the suspended material, there was also a decrease in the rate of clearing of the suspensions. For most experiments using plankton cells, however, he found the rates constant or increasing during the tests. In many instances he reports the rate at the end as being greater than at the start.

In our observations on the rate of clearing of suspended phytoplankton cells by the scallop we found, as did Jørgensen for *Mytilus,* an increasing rate of clearing after the start of observations. This was followed by a time during which the rate was relatively constant. However, in our experiments, as we continued the observations further there was a lessening of the rate of removal of phytoplankton cells similar to the change observed by Jørgensen when suspended graphite particles were used. Later, if the observations were still further continued, there was an increase in the number of cells in suspension.

The pelecypods are able to change the efficiency of their gills as a filtering organ. They form a mucous coat over the gill surface which results in efficient particle retention. The importance of the mucous coating in the feeding activity of lamellibranchs has been stressed by MacGinitie (1941). There is also a possible change in pore size by regulation of the ostea of the gills. Galtsoff (1928) observed that a great number of bacteria added to the sea water were not retained by the gills of the oyster. Similar findings of variations in the retention of bacteria by oysters were reported in more recent work using millipore filters (Galtsoff and Arcisz, 1953). Loosanoff and Engle (1947) observed great variations in the numbers of *Chlorella, Nitzschia,* and *Euglena* removed by the gills of oysters at different times. Jørgensen (1949a) interprets the changes he observed in the clearing of graphite suspensions by *Mytilus* as probably due to the change in efficiency of the gill with a stopping of the secretion of mucus if the particles in suspension were unsuitable for food or the animal disturbed. The efficient removal of the phytoplankton cells was explained in the light of an entrapment in the mucous sheet. Jørgensen and Goldberg (1953) present data showing that the graphite particles used in their experiments were retained almost completely in the filtering organs of the lamellibranchs and the ascidians tested.

In our experiments with the scallop, it seems that the changes in the rate of clearing of the suspensions of phytoplankton cells probably were directly related to the efficiency of the gills. There was an apparent complete removal of the cells from the filtered water after the scallops had become adjusted to the conditions of the experiment. It seems likely that there was formation of a mucous coat which allowed complete retention of the cells in the gills. As the experiments progressed, there was a return from the mucus to the suspension of cells previously removed. This return took place in increasing amount. There may also have been a lessening in the efficiency of the gills in removing suspended cells. It would be unwise to ignore entirely the possibility that the conditions of experimental arrangement had no influence on the observed changes in the rate of clearing of the suspensions. It is almost impossible to have the exact mixing of the filtered water with the unfiltered suspension required to present the scallop with the properly diluted suspension each moment.

The filtration rate may not indicate a true feeding rate, for removal of the cells from the suspension may not result in ingestion by the scallop. Considerable work remains to be done on the role of the mucous coat in filtration and feeding activities. The factors concerned with changes in the secretion of mucus and those governing sorting for ingestion of filtered material need further investigating.

The complete removal of the suspended plankton cells from the water filtered by the scallop allows the use of measurements of the rate of particle reduction to serve as a means of calculating the rate of water propulsion. This indirect method is valuable when methods of direct measurement of the flow are not feasible.

There were no apparent differences in the rate of water filtration of the scallops when immersed in suspensions of phytoplankton cells of different concentrations. Loosanoff and Engle (1947) found that the water filtration of the oyster was reduced when exposed to heavy suspensions of micro-organisms. They observed no effects, however, when the concentrations were light and more comparable to those which may be expected to occur in natural marine waters. Although the threshold concentration for effect varied with the size of the species employed, the values found were high. They reported that oysters were able to reduce the population of plankton to concentrations below this threshold rather rapidly in small containers and then were able to clear the suspension normally. It is conceivable that the concentrations used in our experiments exceeded the effective concentration threshold that would interfere with the rate of water filtration of the scallops and the small volume of the suspensions used enabled the scallops to reduce the cell numbers rapidly to a point below this threshold. If such were the case, we would have failed to observe the effects of concentrations above this threshold on the rate of water filtration. It would be possible to increase the volume of the experimental suspension greatly and provide adequate mixing. The scallops then would reduce the population of plankton cells more slowly and be exposed to high concentrations for greater times. The use of radioactive plankton would still allow the measurement of slight changes in cell numbers. It would be interesting to observe if thresholds of cell concentrations affecting the filtration rate existed that were within those concentrations that the scallop might encounter in nature. In low densities the rates were not markedly increased or decreased over those in higher concentrations. This is in agreement with Jørgensen's observation (Jørgensen, 1952) that filtration rates were not influenced by low concentrations of available food in lamellibranchs and ascidians.

In our observations the scallops were found to have a rather high rate of water propulsion. This rate was greater for the larger scallops than for the smaller. Although the large scallops pumped greater quantities of water through the gills than did the small ones, the average pumping rate per gram of tissue in the larger was somewhat less. Our observations were based on a single year class followed as they grew to an age of 12 to 14 months. During this time there was, of course, development of ripe gametes, which may influence the rate as measured for the larger scallops, causing it to be somewhat high. Similar relationships of rate of pumping to size were reported for mussels by Jørgensen (1943, 1949a), Fox, Sverdrup and Cunningham (1937), Willemsen (1952), and Rao (1953).

If we consider the data available, it appears that the adult oyster (*Crassostrea virginica*) of about 4 or 5 years of age at summer temperatures probably has a water propulsion rate of about 12 to 14 liters per hour (Galtsoff, Chipman, Engle and

Calderwood, 1947; Loosanoff and Nomejko, 1946; and Jørgensen, 1952). Although actual data are not available, the oysters used probably weighed somewhat more than 12 to 14 grams. This would mean that the oyster has a rate of water propulsion a little less than one liter per hour per gram of body weight. This is about the rate observed by us for the bay scallop. For the large mussel, *Mytilus californianus*, Rao (1953) shows data indicating that at 20° C. the animals from 30 to 70 grams had a pumping rate of 5 to 6 liters per hour, and a rate per gram of only 0.1 and 0.2 liter per hour. Evidently this animal has a much lower rate of water propulsion than either the bay scallop or oyster. For *Mytilus edulis*, the rates per animal for small individuals at 22° were about 0.16 liter per hour and for larger from 0.3 to 0.4 liter per hour (Jørgensen, 1949a). For large individuals (70 to 80 mm. in length), at temperatures from 11.8 to 14.7° C. an average of 1.8 liters per hour was found by Willemsen (1952). It seems likely that *Mytilus edulis* may have a rate similar to that of *Mytilus californianus*. The cockle, *Cardium edule*, was found by Willemsen (1952) to have a rate of about 0.5 liter per hour for individuals of 30 to 40 mm. in length at temperatures from 17.3 to 19.5° C. This would indicate that sea mussels and cockles have a rate of water propulsion per gram much less than either the bay scallop or oyster.

Jørgensen (1952) has related the filtration rates of several filter-feeding invertebrates to their oxygen consumption. According to his findings there is a quite uniform ratio between the two in all such forms. In view of the fact that the bay scallop has a rapid rate of growth and is a very active animal, it is not surprising that the rate of water filtration is rather high. The oxygen consumption has not been determined, but it is likely to be also high, and may have the same relation to filtration rate that Jørgensen found for related animals.

SUMMARY

1. The water filtration by the bay scallop, *Pecten irradians*, was studied by following the clearing of suspensions of plankton cells that had been made radioactive. The use of radioactivity measurement techniques for such studies of the rate of water propulsion of filter-feeding invertebrates by the indirect method allows detection of slight changes in cell numbers in dilute suspensions and is advantageous for investigation of the feeding activities of lamellibranch molluscs.

2. The scallops were observed to filter the water passed through their gills very efficiently with apparently complete retention of *Chlamydomonas* and *Nitzschia* cells after adjustment to the immersion in the suspensions. The rapid rate of decrease of suspended plankton was not continued, however, for there was evidence of a decrease in the efficiency of the gills and increasing return to the suspension of phytoplankton cells previously removed.

3. The bay scallop has a relatively high rate of water propulsion, probably correlated with its rapid rate of growth and active mode of life. The average rate for small scallops, 38–44 mm. in length, was 3.26 liters per hour. The largest scallops, about 12 to 14 months of age and measuring 64–65 mm. in length, averaged 14.72 liters per hour. The maximum rate observed was 25.4 liters per hour. The smaller scallops had a rate of about one liter per hour per gram of tissue, whereas the older scallops pumped an average of about 0.7 liter per hour per gram.

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ON THE DISTRIBUTION OF A MARINE CLADOCERAN, PENILIA AVIROSTRIS DANA (CRUSTACEA, BRANCHIOPODA), WITH A NOTE ON ITS REPORTED BIOLUMINESCENCE

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The marine cladoceran, *Penilia avirostris* Dana, is a member of the surface plankton found in warm and temperate coastal waters at widely scattered points throughout the world. Its recent regular appearance in the waters around Woods Hole, Massachusetts, far to the north of its previously supposed temperature limits, has prompted the writing of the present paper. From a survey of the literature it was found that the reported distribution of *Penilia* poses a number of problems of exceptional interest. These problems are here discussed, and the results are reported of certain observations made on living specimens of *Penilia* at Woods Hole. No very definite conclusions have been reached, but it is hoped that other investigators will continue the study, since *Penilia* would appear to be an organism offering certain special advantages to the student of plankton distribution.

Colonization of the Sea from Fresh Water

Of all planktonic marine organisms *Penilia* is perhaps the easiest to identify, for it is the sole marine representative of the cladoceran tribe Ctenopoda, characterized by six similar pairs of filter-feeding trunk appendages enclosed by a bivalve carapace. Indeed even in the related tribe Anomopoda (in which the trunk appendages are not all alike) there are no marine representatives, other than *Bosmina maritima,* found only in the Baltic. Two other cladoceran genera, *Podon* and *Evadne,* are marine, but are members of the aberrant family Polyphemidae, in which the much reduced carapace does not cover the somewhat leg-like trunk appendages. At the present time only a single species of *Penilia* is recognized (see Steuer, 1933a), so that the task of picking *Penilia* out from a plankton sample can be assigned to anyone capable of using a microscope.

Among the many hundred species of filter-feeding Branchiopoda, *Penilia* alone is truly marine. Yet in the other classes of free-swimming Entomostraca, namely the Branchiura, Ostracoda, and Copepoda, we find a much more even distribution of species between marine and fresh water habitats. One might infer that perhaps it is the possession of leaf-like trunk limbs, or "phyllopodia," which has tended to exclude Branchiopoda from marine environments. Storch (1925) and others have pointed out that the effective shape of phyllopodia, and indeed of other parts of the body in these animals, is dependent on the pressure of the blood in the hemocoel. In a fresh water environment this pressure, or state of turgor, can be maintained by a simple osmotic inflow of water. In salt water, maintenance of the turgor would be more difficult. Despite this fact, however, there are a few species, in each of the four orders of Branchiopoda (Anostraca, Notostraca, Conchostraca, and Cladocera), which have colonized saline pools (see, for example: Mathias, 1937; Packard, 1883, p. 369; Parenzan, 1932). In the case of the anostracan, *Artemia,* Kuenen (1939) has shown that the blood is always hypotonic to the surrounding saline medium; the turgor of the blood in this animal has to be maintained by an active transfer of water against the osmotic gradient; if the animal becomes unhealthy it soon loses its plump appearance and shrinks in volume. In the case of the cladoceran, *Daphnia magna,* which can be acclimatized to quite high salinities, the blood is kept hypertonic to the surrounding medium, thus favoring an osmotic inflow of water (Krogh, 1939).

Although thus able to withstand high salinities, these Branchiopoda which can live in saline pools have never been successful in colonizing the sea. They are adapted to life in shallow, often temporary waters, and seem unable to survive in the very different conditions met with in the ocean.

The ancestor of *Penilia* must have migrated from fresh water to the sea through some favorable river estuary. Its closest relatives, including especially the genus *Sida* (see Lochhead, 1936–7), live chiefly in lakes rather than in small pools. As in other filter-feeding Branchiopoda, the shape of the phyllopodia and other parts of the body in *Penilia* doubtless depends on the maintenance of internal turgor. Specimens of *Penilia* which have sunk to the bottom of a container have a bedraggled appearance, in contrast to the plump specimens still swimming actively in the water above. It is not known whether the internal turgor is achieved by an active transport of water, as in *Artemia,* or by keeping the blood hypertonic, as in *Daphnia.* Fox (1952) reports that *Penilia,* in common with other small Crustacea, constantly ingests water, through both the mouth and the anus. Much of the ingested water seems to be absorbed, but Fox doubts that this is the primary mechanism for maintenance of internal turgor.

Podon and *Evadne* do not have phyllopodia, their limbs being more like those of ostracods. It may be that the shape of these animals is not so dependent on internal turgor as in other Branchiopoda. In this connection it is perhaps significant that in the family Polyphemidae, to which *Podon* and *Evadne* belong, there are more species in salt than in fresh water.

Penilia, Podon and *Evadne* share in common two features of the reproductive system that are not found in a majority of other Cladocera. Perhaps, therefore, these are features that in some way have proved helpful in the colonization of the sea, though in just what way is not very evident. The fertilized, resting eggs have thick shells, and are not enclosed in a protective "ephippium." The eggs which develop parthenogenetically are small, almost devoid of yolk, and during embryonic development increase greatly in size by absorption of a nutritive fluid secreted by the mother into the brood sac. This last feature is characteristic of both fresh water and marine Polyphemidae, but it is not known for any Ctenopoda other than *Penilia* (Storch, 1925; Sudler, 1899). Perhaps it is no more than a coincidence that the same feature is found also in the anomopod genus *Moina,* species of which are among the Cladocera most frequently encountered in saline pools.

SPORADIC AND DISCONTINUOUS DISTRIBUTION

Information available to date indicates that *Penilia* has an extremely sporadic and discontinuous distribution. It has been recorded from the shores of every continent, frequently in tremendous numbers, but from widely separated points and often only during a single season. At Beaufort, North Carolina, Sudler found *Penilia* in June, 1896, in such numbers as to clog a net towed for only a few meters through the water. Not until 1931 was *Penilia* again recorded from our Atlantic coast, when it was taken off New Jersey (Bigelow and Sears, 1939). Dr. R. E. Coker very kindly informs me that he later found a single specimen in plankton taken outside Beaufort Inlet, in January, 1949, and that a number of specimens were taken there towards the end of 1953. At Naples, Italy, *Penilia* was unknown until 1922, when Caroli found it throughout the summer, the population becoming so large in late August as to form practically the entire surface plankton. In this case the population became established, and *Penilia* has since reappeared at Naples in large numbers each summer (Cattley and Harding, 1949). In the waters off Sydney, Australia, *Penilia* has been recorded on various occasions since 1894, and recently its volume there was so great (0.85 ml./m.^{3}) as to constitute the richest haul of non-salp plankton taken in Australian waters (Sheard, 1949). This compares with the best hauls of non-salp zooplankton commonly taken in temperate northern waters, although volumes of over 5 ml./m.^{3} have been recorded (Clarke and Bishop, 1948).

Further examples could be cited, but enough has been said to show how suddenly *Penilia* may appear, and the abundance it may attain. The periods of abundance are always associated with a high rate of ovoviviparous parthenogenesis, as in other Cladocera, but the fluctuations must be correlated with as yet unknown environmental factors. Since *Penilia* can be so easily identified, has a direct development uncomplicated by larval stages, and has feeding habits that are reasonably well known (Lochhead, 1936–7), it should be a favorable species for a study of factors underlying such seasonal fluctuations in abundance as are discussed by Sears and Clarke (1940).

An incomplete summary of the discontinuous world distribution of *Penilia* has been given by di Caporiacco (1938). Additional records will be found in some of the references listed at the end of the present paper. No simple explanation is evident for this discontinuous distribution, nor for the sporadic appearances of *Penilia*. In the case of fresh water Cladocera, examples of the same kind can be explained by assuming a chance distribution of resting eggs, especially on the feet of birds, to isolated bodies of water. Coastal waters of the oceans are, however, continuous, and opportunity for the resting eggs of *Penilia* to become attached to the feet of birds would not appear to be great. The resting eggs of Ctenopoda and Polyphemidae are generally said to be cast loose into the water. Weismann (1877) found those of fresh water species lying at the bottom of aquaria. It also has been supposed that the resting eggs of *Penilia*, *Podon*, and *Evadne* sink in sea water (Bigelow and Sears, 1939; Friedrich, 1952). However, Jorgensen (1933–4) found the resting eggs of *Evadne nordmanni* in surface plankton, enclosed in the parental exuviae. She suggests that they may not sink as rapidly as had been supposed.

They might then be dispersed widely by currents, in spray by the wind, and to beaches where they could be picked up on the feet of birds. It seems unlikely, however, that such a condition holds true for *Penilia*. Caroli (1924), who followed throughout a summer a population of *Penilia* with a relatively high output of resting eggs, never saw these eggs enclosed within exuviae. He concluded that the eggs probably sink to the bottom. Possibly the sudden appearance of *Penilia* in new localities occurs only when a few recently released resting eggs, that have not yet had time to sink, chance to have been caught up in wind-driven spray.

More difficult to explain is the apparent absence of *Penilia* from many intermediate locations. This might seem to indicate that *Penilia* has only recently invaded the sea. If that were true, however, one would expect to find *Penilia* especially abundant in river estuaries, whereas it more usually is found in open coastal waters. Perhaps unsuspected environmental factors have excluded *Penilia* from certain areas. Or it may be that populations in some areas have died out during parthenogenetic reproduction through the action of dominant or semi-dominant lethal genes. Instances of this kind have been recorded by Banta and Wood (1939) for some fresh water Cladocera. Banta (1939) notes that there are wild populations of certain fresh water Cladocera which are never known to reproduce other than by ovoviviparous parthenogenesis. In one case he found a mutant stock in which both resting and "summer" eggs were produced by parthenogenesis. It may well be that in some areas *Penilia* reproduces only by parthenogenesis, probably then without the production of resting eggs. Information on this question is very scanty, since most of the reports dealing with *Penilia* fail to mention the sexes taken or the type of reproduction observed. Steuer (1933a, 1933b) stated that males were at that time known only from Hong Kong, Naples, the Adriatic, and the Black Sea. In the literature I have found only one additional record that seems to refer to the occurrence of males. Dakin and Colefax (1940) state that off Sydney, Australia, *Penilia* was "found breeding in April and May." Since this was at the end of the season, and maximum numbers of *Penilia* were noted in January and February, it seems probable that the statement was meant to refer to bisexual reproduction. One more locality for the occurrence of males and resting eggs can now be added, namely, Woods Hole, Massachusetts, where in August, 1953, I found about one or two per cent of the population of *Penilia* to be males, and a lower percentage to be females bearing resting eggs.

Obviously, further records are needed before any conclusions can be drawn as to the prevalence of males and resting eggs in different localities. It would be helpful if those who record the presence of *Penilia* would always take care to state what types of individual were found. Steuer (1933b) figures juvenile instars and the three types of adult, namely, males, females with one or two resting eggs, and parthenogenetic females with "summer" eggs or embryos. "Summer" eggs which have not yet developed into embryos are much smaller than resting eggs, and in most cases are more numerous. Persons not having access to Steuer's paper should be able to identify the different types of adult by reference to Figure 1, which shows a female bearing a single resting egg, and a male one instar prior to maturity. Mature males have penes over twice the length of those shown, and antennules reaching to the posterior border of the carapace. Males two instars prior to maturity can be recognized

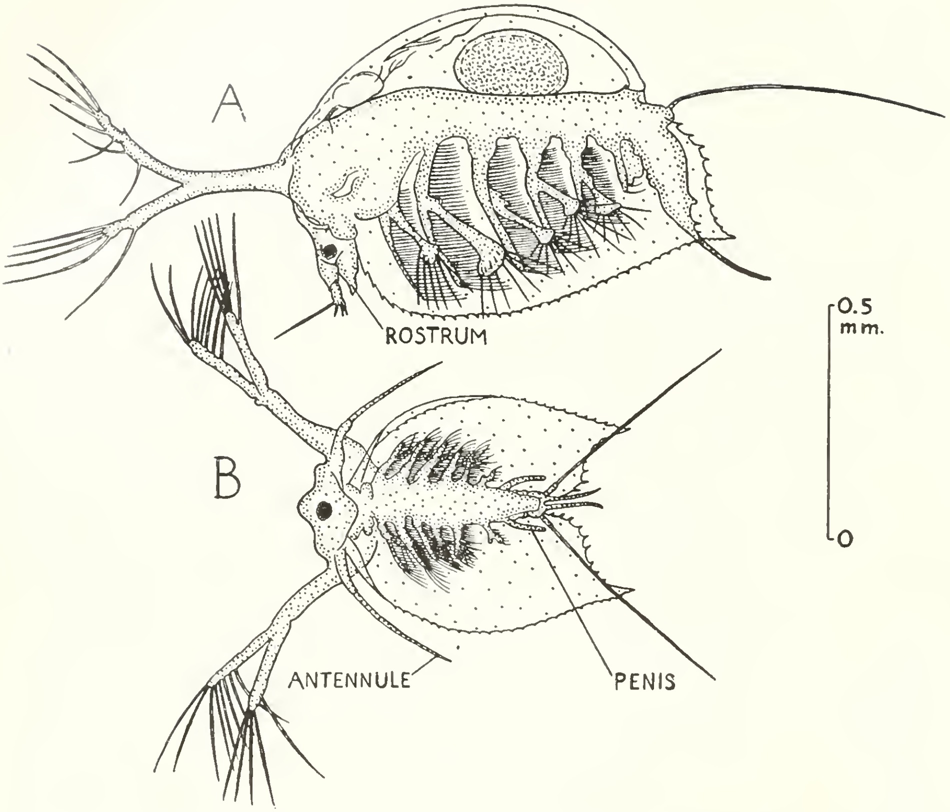

Figure 1. *Penilia avirostris* Dana, from Woods Hole, Massachusetts, August, 1953. Sketched from preserved specimens, a camera lucida being used for the main outlines. A, mature female, with a single resting egg in the brood sac; B, male one instar prior to maturity; the shell valves are here much wider apart than they would be in a living specimen.

by small rudiments of the penes, antennules already twice the length of those in a female, and, as in all males, by the absence of a pointed rostrum.

Restriction to Coastal Waters

The restriction of *Penilia* to coastal waters is another feature for which no definite explanation can be offered at present. The greatest distances from shore at which *Penilia* has been taken seem to be those reported by Bigelow and Sears (1939) (about 60 km. off the coast of New Jersey) and by Marukawa (1921) (either about 230 km. or about 310 km. east of Japan). Neither temperature nor salinity would appear to be a factor excluding *Penilia* from the open sea. Some authors have spoken of *Penilia* as a predominantly brackish water form (*e.g.*, Gibitz, 1922). There has even been a single isolated record of *Penilia* from fresh water (Krämer, 1895). However, most of the records of *Penilia* have been from localities with salinities above 32‰. Leder (1915) found *Penilia* in salinities from 10‰

to 37‰. Della Croce (1952) found maximum numbers of *Penilia* in the Gulf of Genoa when the salinity was above 38.3‰. Gurney (1927–9) found *Penilia* abundant in the Gulf of Suez where the salinity was 43.8‰, and even recorded it from a station in the Suez Canal where the salinity was 49‰ (salinities taken from Fox, 1926–9). It is evident that the salinity of the open ocean lies well within the range normally tolerated by *Penilia*. At low temperatures, however, the resistance to high salinities may be reduced, in accordance with Moore and Kitching's (1939) "idea of modified tolerance." Thus Dakin and Colefax (1940) found *Penilia* four miles out to sea off Sydney, Australia, reaching maximum abundance in summer, yet also found it to be sometimes exceedingly abundant in winter in the more estuarine catches. This is despite the fact that minimum surface water temperatures were lower in the estuary (12.3° C.) than at the offshore station (15–16° C.) (Dakin and Colefax, 1935). Salinity at the offshore station was 35.4–35.8‰; that within the estuary was unfortunately not given.

Perhaps the restriction of *Penilia* to coastal waters is a result of a shortage in the open sea of fine detritus and minute organisms, under 5 μ in diameter, on which *Penilia* feeds (Lochhead, 1936–7). However, other animals requiring equally fine food do live successfully in the open ocean.

If it should be found that populations of *Penilia* must everywhere be renewed each year from resting eggs, and if these eggs sink to the bottom, as seems probable, then the restriction of *Penilia* to waters of moderate depth would be understandable. However, in tropical regions one would expect at least some populations to be successful in reproducing throughout the year exclusively by ovoviviparous parthenogenesis.

RESTRICTION TO SURFACE WATERS

Apparently *Penilia* always occurs relatively close to the surface. Gamulin (1948–9) records it from depths of 20–70 meters in the Adriatic; Motoda and Anraku (1952) found it only at depths of 10–20 meters in "Funka Bay," Hokkaido, Japan (apparently a synonym for Volcano Bay, judging from a map (Fig. 4) in Kawasaki *et al.*, 1952–3). Nearly all other records seem to have been from surface plankton tows.

I have tested the reactions to light of specimens of *Penilia* taken at Woods Hole, with purely negative results. When illuminated only from one side by a bright, horizontal beam of light from a spot lamp, *Penilia* continued to swim in various directions in all parts of a jar, whereas copepods in the same jar quickly aggregated towards the light. Specimens of *Penilia* also swam at random, both upwards and downwards, when illuminated only from above; the spot lamp was placed 23 cm. above a small tube, holding a column of water 60 mm. high and 18 mm. in diameter, standing on a dull black surface. Copepods tested under these same conditions aggregated immediately towards either the top or the bottom of the tube, depending on the species, in contrast to *Penilia* which showed no such tendency even over periods of up to fifteen minutes. In both the horizontal and the vertical beams of light results were the same with light-adapted *Penilia* and with *Penilia* which had been kept in total darkness for periods of from one to twelve hours. In all cases the specimens spent much time floating suspended in the water, swimming in sudden spurts at irregular intervals, as is characteristic for the species. Neither when floating nor when actively swimming could the specimens be seen to show any type of dorsal light response to the direction of illumination. It may be concluded that the restriction of *Penilia* to surface waters would appear not to be in response to light.

Nor does it seem that this restriction depends on a response to pressure, of the type discovered in crab larvae by Hardy and Bainbridge (1951). To test for such a response, specimens of *Penilia* which had been kept in the dark for twelve hours were transferred to a 10-ml. syringe, over the lower opening of which a piece of wax paper had been tightly tied. The level of sea water was adjusted up to the mark and the plunger then very slowly pushed down until all air had been expelled. With the lower end of the syringe resting on a table, pressure on the plunger was now increased steadily by hand up to a maximum later found equal to about 10.5 kilograms. Since the internal diameter of the syringe was 14 mm., it can be computed that the maximum water pressure attained, additional to atmospheric pressure, was approximately 6.8 kg./cm.^{2}, corresponding to a depth in the sea of about 67 meters. The specimens of *Penilia* in the syringe were observed against a brightly lighted dark background. At all pressures they continued to swim normally, showing no tendency to aggregate at any particular level in the column of water.

It must remain for the future to determine what factors may be responsible for the restriction of *Penilia* to surface waters. Perhaps a response to temperature is involved, or perhaps under some conditions the animals display negative geotaxis. However, no evident responses to gravity were seen in the specimens observed in vertical tubes.

Distribution as Related to Temperature

The distribution of *Penilia* towards the poles is limited by temperature. Calman (1917–23) stated that the mean annual surface isotherms of 18° C. would include all localities where *Penilia* had been found, except for Trieste, the Black Sea, and possibly New Zealand. This statement remained unchallenged for many years, and it therefore was with considerable surprise that in 1944 I recognized what appeared to be a drawing of *Penilia* among some sketches of plankton organisms turned in by a student in the Invertebrate course of the Marine Biological Laboratory, Woods Hole, Massachusetts. The specimen was dead when found, and had been thrown away after being drawn. In 1946 a plankton sample collected for the Invertebrate course contained considerable numbers of *Penilia*, which I was able to examine alive. The same was true in 1948, 1949, 1951, 1952, and 1953. The samples each year were collected from the surface waters of Great Harbor, Woods Hole, between August 23rd and August 31st, with additional samples in 1953 from July 29th to September 1st. No especial search for *Penilia* was made in any year, so that it is quite likely that *Penilia* has been present in Woods Hole waters each year since 1944. Mr. A. Fleminger, a graduate student at Harvard University, has kindly turned over to me some specimens of *Penilia* taken in surface plankton from Menemsha Bight, Martha's Vineyard, about 20 km. S.S.W. of Woods Hole, July 25, 1951 (one specimen) and September 1, 1951 (25 specimens). Deevey (1952a, 1952b) has recorded the presence of *Penilia* in Block Island Sound, about 90 km. W.S.W. of Woods Hole, in 1943, 1944, 1945, and 1949.

One of her hauls in September, 1943, yielded about 2900 *Penilia* per fifteen-minute tow; another haul in October, 1949, yielded 900 *Penilia* per cubic meter.

Thus it is evident that at least since 1943 *Penilia* has occurred, often in large numbers, off the southern coast of New England. It must be a recent arrival, since it is not mentioned in any of the plankton surveys of the region made prior to 1943. As already noted, most of the specimens seen by me have been females, reproducing when adult by ovoviviparous parthenogenesis. One large specimen contained 13 embryos, a number which exceeds both the observed and the predicted maxima given by Steuer (1933b).

To gain more precise knowledge of the lower temperature limits controlling the distribution of *Penilia,* I have gathered together the information given in Table I. In my search of the literature I have not been able to find any records of *Penilia* from localities farther north or farther south than those shown in this table. It will be seen at once that the mean annual surface temperature at Woods Hole lies far below the limit of 18° proposed by Calman, or the limit of 17° more recently proposed by Fuller (1950). However, it will also be seen that *Penilia* has been taken from four other localities with mean annual surface temperatures well below these limits.

Actually, as pointed out by Hutchins (1947), the poleward distribution of marine organisms is controlled, not by the mean annual surface temperature, but by the lowest temperature tolerated in winter and by the minimum temperature necessary for reproduction in summer. Neither of these temperature limits is known for *Penilia.* Fuller (1950) thought that *Penilia* requires a temperature of at least 18–20° during a period long enough to permit reproduction. This suggestion agrees well with most of the records that we have for *Penilia.* However, Cattley and Harding (1949) found *Penilia* reproducing in October off the coast of Holland, in water of 16.68°. Possibly on occasion even lower temperatures would suffice, judging from the statement of Dakin and Colefax (1940) that in estuarine waters near Sydney, Australia, *Penilia* sometimes becomes exceedingly abundant in winter, when surface water temperatures of about 12–15° might be expected (Dakin and Colefax, 1935).

At Woods Hole the surface water temperatures in summer should more than suffice for the reproduction of *Penilia* (means for July, August, and September in the period 1945–1951: 21.3°, 21.8°, and 20.0°). The temperatures encountered in winter, however, are much lower at Woods Hole than in most of the other localities from which *Penilia* has been recorded. Reference to Table I will show that only in three of the other localities are the surface water temperatures in winter nearly as low as at Woods Hole.

In regions where the population of *Penilia* has been followed through a season, it is known that during the autumn the numbers gradually decrease to zero. The lowest temperature at which a surviving adult has been taken appears to be 8.7° (Steuer, 1933b). Usually all specimens are gone at temperatures well above that value. Survival in colder temperatures presumably must be in the form of resting eggs.

Of the localities listed in Table I, only Woods Hole, Odessa, and Auckland represent regions where *Penilia* has become well established. There are three reports of its occurrence in northern Japanese waters. The other localities represent simply isolated records which could perhaps be looked on as abnormal oc-

TABLE I

Locality	*Penilia* reported by	Mean surf. water temps. (° C.)			Authority for temperatures
		Coldest month	Warmest month	Annual	
Woods Hole, 1945–1951	Present paper	Feb., 0.7°	Aug., 21.8°	11.5°	1
Woods Hole, 35 years of period 1885–1941	No report	Feb., −0.2°	Aug., 21.1°	10.4°	2
Lat. 40°N., 175 km. south of Woods Hole	No report	March, 6.4°	Aug., 20.6°	13.0°	3
Dutch coast, 52° 22′N., 4° 20′E.	Cattley and Harding	Feb., 3.5°	Aug., 17.5°	10.2°	4
Near Odessa, Black Sea	Zagorowsky (see Steuer, 1933b)	Feb., 1.1°	July–Aug., 22.2°	13.5°	3
Muroran, Hokkaido, Japan	Tanita, Kato, and Okuda	March, 2.2°	Aug., 21.7°	11.0°	3
Near Cape Town, S. Africa, 35° 13′S., 20° 17′W.	Rammner	July, 15.2°	Jan., 20.6°	18.2°	3
Cook Strait, New Zealand	Steuer, 1933a	July, 10.0°	Feb., 16.1°	13.2°	5
Auckland, New Zealand	Krämer. Fuller.	July, 12.1°	Feb., 21.0°	16.7°	6

Authorities for temperatures in Table I

1. Calculated from monthly mean surface temperatures in the years 1945–1951 at the Woods Hole Oceanographic Institution dock, consulted through the kindness of Mr. Dean F. Bumpus. Closely similar figures for the years 1945–1950 have been published by the U. S. Coast and Geodetic Survey (Publ. No. TW-1, 1951).

2. Calculated from monthly and annual mean surface temperature records, very kindly supplied by Mr. John B. Colton, U. S. Fish and Wildlife Service, Woods Hole. Records are not available for the period 1915–1932, nor for a few other scattered years.

3. Figures for each month read from: World Atlas of Sea Surface Temperatures, H.O. No. 225, Hydrographic Office, U. S. Navy, Washington, D. C.: 50 p., 1944.

4. Figures for each month read from: Böhnecke, G., and G. Dietrich, 1951. Monatskarten der Oberflächentemperatur für die Nord- und Ostsee und die angrenzenden Gewässer. Deutsches Hydrographisches Institut, Hamburg: 18 p.

5. Figures for each month read from charts in: Monthly sea surface temperatures of Australian and New Zealand waters; Marine Branch Meteorol. Office, Air Ministry, London; M.O.M. 482: 14 p., 1945.

6. Calculated from monthly mean surface temperatures for each of the years ending April, 1929, to April, 1941, in: Report on Fisheries, New Zealand Marine Dept., years ending March, 1931, to March, 1941.

currences. With this in mind it might appear that only where the mean surface temperature in the warmest month reaches or exceeds 21° can *Penilia* become permanently established. Perhaps such warm temperatures are necessary to permit the building up of a minimal population density, sufficient to assure the survival of at least some individuals, or resting eggs, through the winter.

In localities such as Woods Hole and Odessa, where temperatures are adequate in summer but very low in winter, one must consider the possibility of repopulation each spring from warmer waters to the south. This probably would have to be by currents, if we accept the assumption, presented earlier, that resting eggs can be caught up in wind-driven spray only when newly released, which would be chiefly in late summer and autumn. Unfortunately our knowledge of non-tidal surface currents south of the New England coast is very incomplete. The complexity of the situation is well presented by Haight (1942). More recent information is given by Zinn (1950), and an analysis of the complex tidal currents near Woods Hole has been presented by Redfield (1953). Rather detailed charts of non-tidal surface currents in the waters east of the United States were published for each month of the year by the German "Oberkommando der Kriegsmarine" (1943), but must include much that is based only on conjecture. In general, it would seem that coastal organisms to the south of New York would stand little chance of being brought to New England waters; organisms from the Gulf Stream, directly to the south of New England, such as the siphonophore *Physalia*, certainly are brought to the New England coast in some summers, but the existence every year of such currents from the south is highly doubtful.

Table I shows that if *Penilia* occurred just beyond the continental shelf to the south of Woods Hole, it would encounter minimal winter temperatures of about 6.4°. But summer temperatures there might not be adequate for a high rate of reproduction; the presence of *Penilia* so far from land would not be expected; and the annual occurrence of suitable currents to bring the organisms to the coast seems rather unlikely.

Thus it appears probable that *Penilia* is now a year-round inhabitant of Woods Hole waters. If so, the resting eggs must be able to withstand winter temperatures down to below zero (the mean surface temperature at Woods Hole for January, 1948, was − 1.2°; a temperature of − 2.8° was recorded on one occasion in 1945). In effect this would mean that the only temperature requirement for *Penilia* is a summer temperature of perhaps 21° or above, with lower temperatures possible in regions where a high population density is not necessary to assure survival through the winter.

From Table I it will be seen that water temperatures at Woods Hole in the period 1945–1951 averaged slightly higher than in previous years. However, the facts stated above make it unlikely that the slightly colder winters of the past were responsible for the absence of *Penilia* from New England waters.

Discussion

It must remain for future investigation to provide more certain data, and in particular to discover an explanation for the apparent absence of *Penilia* from so many regions that would appear favorable for its development. Doubtless its occurrence has frequently been overlooked, but in regions where the plankton has been intensively studied one would expect it to be reported if present. Thus at both Woods Hole and Naples, *Penilia* is now abundant, but would seem to have been absent at least for many years in the past.

Possibly such sudden inroads into new areas have been preceded by a process of genetical pre-adaptation to wider temperature limits, as reported for fresh water Cladocera by Brown (1929). (See also Johnson, 1952.) Perhaps, too, the production of resting eggs by *Penilia* occurs only in a few genetic stocks, of localized distribution. Reproduction by parthenogenesis alone might lead to local extinction, as noted earlier, and the failure to produce resting eggs presumably would prevent colonization of areas where water temperatures are low in winter. Many other interesting possibilities could be suggested. The whole question of how the population cycle of *Penilia* may be related to the population cycles of other planktonic organisms remains virtually an untouched field. Some data relative to this problem will be found in the papers of Dakin and Colefax (1933), Deevey (1952b), Massutí Alzamora (1949), and Sheard (1949).

TESTS FOR BIOLUMINESCENCE

A report concerning *Penilia* that would be of considerable interest if it could be confirmed is contained in the paper of Dakin and Colefax (1940), who state that *Penilia* "appears to be one cause of luminescence in our plankton." They add that "no distinct luminous organs have been noticed." In August, 1953, I attempted to repeat Dakin and Colefax's observation, with wholly negative results. Dark-adapted specimens of *Penilia* were picked out from a freshly caught plankton sample, and were observed in the dark in groups of ten or more concentrated in a small volume of water. Stimuli used to provoke luminescence were mechanical stirring with a glass rod, drop by drop addition of formalin, ammonium hydroxide, alcohol, and glycerine, the make and break passage of an electric current at various intensities from an inductorium, and exposure to ammonia vapor, the specimens in this case being supported in air on a piece of bolting silk. These stimuli include the principal ones used by Giesbrecht (1895) in his search for luminescent copepods. In no case did any of the specimens of *Penilia* exhibit bioluminescence. This was in contrast to other unidentified microscopic organisms, which luminesced brightly in samples of the plankton from which all specimens of *Penilia* had been carefully removed.

It seems reasonably certain that *Penilia* is not luminescent at Woods Hole in the month of August. However, Giesbrecht found that copepods at Naples, if they luminesce at all, do so for the most part only in the winter. Dakin and Colefax observed *Penilia* in Australian waters at all times of the year, and perhaps it was in the winter that their specimens seemed to be luminescent. If *Penilia* is self-luminous at times, it is probably unique in this respect within the class Branchiopoda (Harvey, 1952), and it might be cited as an especially interesting example of the general rule that among aquatic organisms the only ones that are luminescent are marine.

SUMMARY

1. *Penilia* has recently become a regular member of the summer plankton off the southern coast of New England, much farther north than would formerly have been expected.

2. Its reproduction in New England waters is mainly by ovoviviparous parthenogenesis, but males also occur, together with females bearing fertilized, resting eggs. There is reason to believe that the species overwinters locally in the form of resting eggs, rather than being brought in each year by wind or currents.

3. The poleward distribution of *Penilia* seems to be limited only by the need of a certain minimum water temperature in summer. Adults can survive at temperatures down to below 9° C., and are able to reproduce at and perhaps below 16° C. But the species has not become permanently established except in areas where summer water temperatures exceed 21° C. The resting eggs probably can survive winter temperatures of below − 2° C.

4. *Penilia* is found in every ocean of the world, often as one of the dominant plankton organisms, yet it is absent from many regions where it might be expected to thrive. Possible reasons for this discontinuous distribution are discussed. Sudden appearances of *Penilia* in new areas perhaps are the result of recently laid, resting eggs becoming caught up in wind-driven spray.

5. The restriction of *Penilia* to coastal waters may be because the resting eggs sink to the bottom. Salinity is not the limiting factor, since *Penilia* has been found in salinities ranging from fresh water to 49‰.

6. *Penilia* shows no obvious responses to light or to pressure which might account for its restriction to surface waters.

7. The fact that *Penilia* is the only filter-feeding branchiopodan to have become truly marine, may possibly be because in sea water it is difficult for members of this group to maintain the necessary turgor within their phyllopodia.

8. A report that *Penilia* is bioluminescent could not be confirmed for specimens examined in August at Woods Hole, Massachusetts. The possibility remains that in some localities it may be luminescent in winter.

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THE FIBRILLAR SYSTEMS OF CILIATES AS REVEALED BY THE ELECTRON MICROSCOPE. II. TETRAHYMENA[1]

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Recently the fibrillar systems of ciliates have attracted the attention of several investigators. This revival of interest has resulted in large measure from an appreciation of the morphogenetic properties of these systems. These properties appear to include self-duplication, genetic continuity, pluripotency and a role in the establishment of the polarity and fine morphology of ciliates (Fauré-Fremiet, 1948a; Lwoff, 1950; Weisz, 1951). Unfortunately, continued progress of these studies has been limited by an inadequate understanding of the micro-structure of ciliate fibrillar systems. However, a clearer knowledge of these structures has recently been obtained from an electron microscope study of *Paramecium* (Metz, Pitelka and Westfall, 1953). The present investigation was undertaken to extend this study to other material.

Tetrahymena was selected for this second effort not only because of the wide popularity of this organism as a laboratory animal but also because it can be cultured readily in the absence of other organisms; its fibrillar morphology has been studied exhaustively within the limits imposed by light optics (most recently by Corliss, 1952a, 1953); its morphogenesis at fission has been examined in detail (Chatton, Lwoff, Lwoff and Monod, 1931; Furgason, 1940); and finally its locomotion appears to involve an acetylcholine-acetylcholinesterase system (Seaman, 1951; Seaman and Houlihan, 1951).

The pellicle and oral anatomy of *Tetrahymena* are found to differ greatly from that of *Paramecium* but the kinety (silver line or neuromotor) systems of the two organisms are basically similar in structure and organization. The kineties of *Tetrahymena*, like those of *Paramecium*, are compound structures composed of discrete units. Each unit consists of a cilium, a kinetosome (ciliary basal body) and a short, tapering, kinetodesmal fibril with periodic structure. As reported briefly elsewhere (Metz, 1953), these units are associated by overlapping of the individual kinetodesmal fibrils to form the kinetodesma or silver line fiber of the light microscopist.

MATERIAL AND METHODS

Tetrahymena gelcii,[3] strain W, was used exclusively in this investigation and the writers are indebted to Dr. G. W. Kidder for the initial culture of this organism. All subsequent cultures were grown in 2% proteose peptone in the absence of other organisms. Three- to seven-day old cultures served as starting material for electron

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[3] Following an exhaustive study Corliss (1952b) concludes that the specific name *pyriformis* should have priority over *gelcii* (Furgason, 1940).

microscope examination. With few exceptions these rich cultures revealed no dividing animals when examined with light optics.

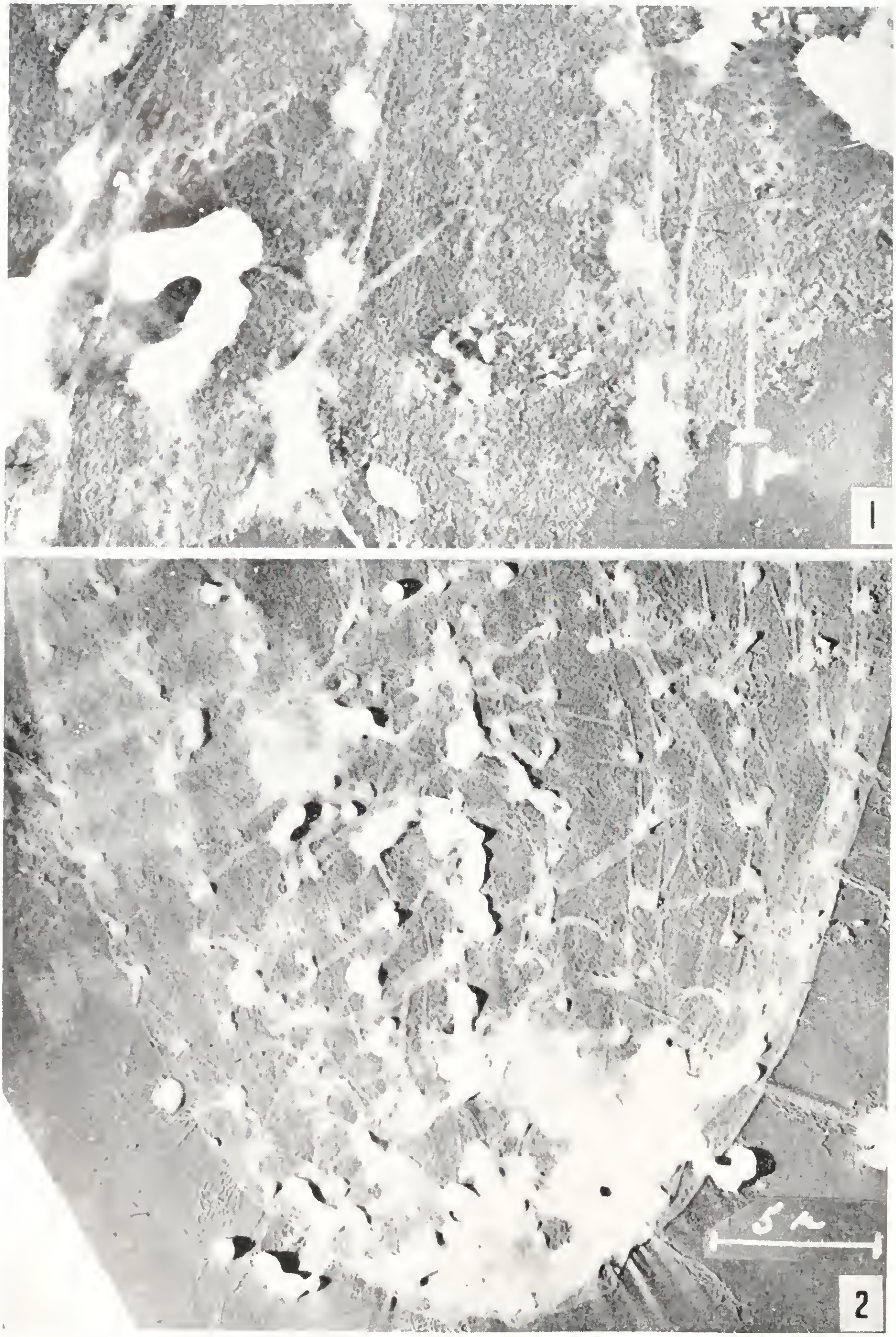
Preparation of the material for electron microscopy

The preparation procedure employed in the *Paramecium* study (Metz, Pitelka and Westfall, 1953) was transferred to *Tetrahymena* with little modification. It consisted of the following steps: (1) fixation, (2) distilled water washing to remove fixative, (3) sonic dissection (9 Kc magnetostriction oscillator, model S-102A, Raytheon Corp.), (4) fractional centrifugation to isolate and concentrate pellicular fragments, (5) mounting (air drying specimens to collodion-coated electron microscope screens) and (6) shadow casting with chromium. The critical dissection, concentration and mounting operations (steps 3, 4 and 5) were followed with phase optics.

Formalin and osmium tetroxide vapor were employed as fixatives. Formalin fixation was achieved by mixing equal volumes of untreated *Tetrahymena* culture and diluted formalin (commercial formalin diluted to 1 15 with distilled water giving a final formalin concentration of 3.3% in the mixture). The mixture was allowed to stand for one to several hours and finally processed as described above. To obtain osmium tetroxide fixation, one to several ml. of *Tetrahymena* culture (with or without previous concentration by centrifugation) were poured into a syracuse or petri dish and exposed to osmium tetroxide vapor in a closed vessel. Following fixation the material was processed as outlined above. The osmium tetroxide material was examined most extensively. Although this method of preparation sometimes gave beautiful preparations, it did not do so consistently. In many cases the fibrillar apparatus appeared to be coated with precipitated cytoplasm and mitochondria when examined with electron optics. Indeed the kineties of *Tetrahymena* appeared to serve as preferential sites for such precipitation and in a number of preparations the primary kinety fibrils were completely obscured by such material. This difficulty was encountered in other forms, notably *Blepharisma* and *Stentor*, in preliminary examinations. This variable behavior may have an important bearing upon the results obtained with the silver impregnation technique (*e.g.*, von Gelei, 1935; Corliss, 1952a). With limited access to the electron microscope it was not possible to standardize the procedure to the point where consistently favorable results were obtained. However, fixation appears to be the critical operation. Prolonged treatment with osmium tetroxide vapor is to be avoided. Just sufficient exposure to kill the organisms, followed by judicious sonic treatment (phase microscope observations at 30-second to one-minute intervals), gave the most satisfactory preparations. Indeed the best specimens were fixed so lightly that the cilia frayed out (Figs. 1, 2) and macronuclear membranes sometimes broke down revealing interesting internal structure. Evidently some fixation is necessary, for all attempts to obtain pellicular fragments by sonic treatment of living animals produced only breis.

Electron microscope examination

The material was examined with an RCA model EMU electron microscope using standard procedures. However, one unusual detail of procedure must be emphasized. This concerns the orientation of the specimen in the electron microscope.



Figures 1–2.

The specimens are fragments of the protozoan surface, they are asymmetrical (three axes of asymmetry, *e.g.* Figs. 1, 6) and their relations to the morphology of the intact animal must be interpreted in terms of this asymmetry. The specimen screen may be mounted in the electron microscope so that the specimen side of the screen faces toward or away from the photographic plate of the instrument and either arrangement is acceptable practice. However, a reversal of symmetry will result in changing from one situation to the other. When the specimen surface of the screen faces away from the plate, normal symmetry relations obtain on the plate; when the specimen surface faces toward the plate, normal symmetry is reversed and a mirror image results. This problem is seldom critical in electron microscopy (Hillier, personal communication) but its importance cannot be over-emphasized in the present instance.[4] It follows that all subsequent photographic manipulations (reversal or "second negative" and final printing) must be performed with equal attention to symmetry.

The screens were always mounted specimen side down in the electron microscope specimen screen holder (facing the photographic plate). Proper orientation was facilitated by using Athene screens (Smethurst High-Light Limited) which have easily recognizable differences in their surfaces.

Limitations of the method

The rather drastic methods of preparation employed introduce certain hazards and limitations to the study. These include the following: (1) artifacts may be introduced by fixation, sonication and drying; (2) observations are usually made on fragments of many organisms selected at random, making it difficult to reconstruct the morphology of a single individual; (3) the sonic dissection method involves removal of parts of the organism under conditions that preclude direct observation. These limitations have been discussed and evaluated in the previous study (Metz, Pitelka and Westfall, 1953). They apply with nearly equal force in the present instance. However, *Tetrahymena* does possess two striking advantages over *Paramecium*. First, when grown in the absence of other organisms, all material observed with the electron microscope may be considered to be a part of the organism or a byproduct of it, if suitable precautions are taken to prevent organic and other contamination during preparation. Second, *Tetrahymena* is of such a size that large, easily recognizable fragments or whole, "eviscerated" animals can be examined with the electron microscope (*e.g.*, Fig. 2). Both of these advantages were exploited in this investigation.

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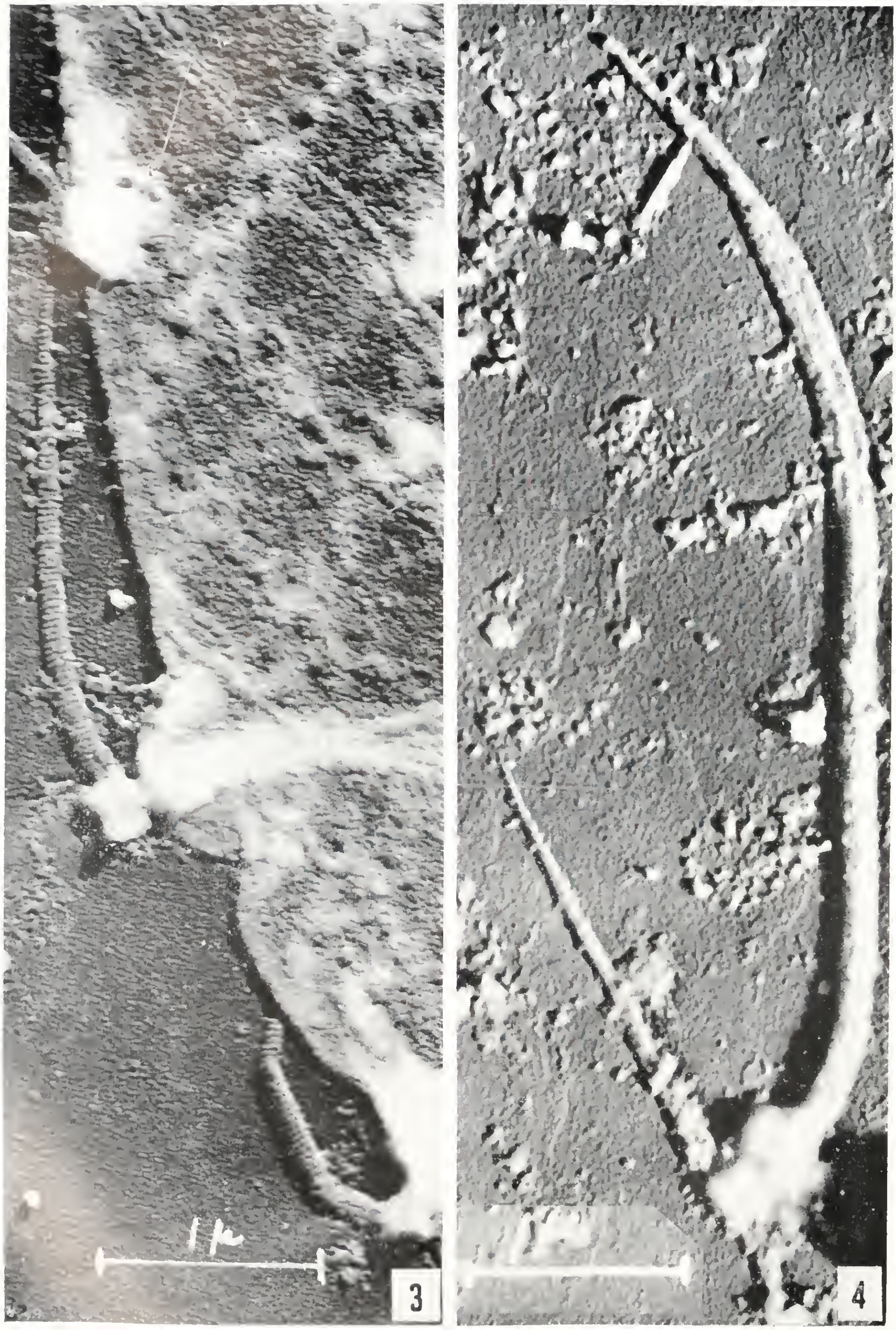
[4] These precautions were followed in the Paramecium study although they were not stressed in that paper.

FIGURE 1. A fragment of pellicle viewed from the inner surface. Segments of three kineties are shown. Each of these consists of several kinety units (3, 4 and 5 units in the right, middle and left kineties, respectively). Each kinetodesmal fibril is seen to arise from a kinetosome and overlap the next unit. Bases of the cilia pass through ring-shaped thickenings in the pellicle. The striated area of the pellicle is seen immediately to the right of the kineties. The chains of ring-shaped thickenings in the pellicle are evident midway between the kineties. The granular area of the pellicle is located to the right of the pellicular chains. Part of a frayed cilium is visible through the pellicle (upper left). Osmium tetroxide fixation.

FIGURE 2. Posterior end of an "eviscerated" animal. The individual kinetodesmal fibrils are directed toward the anterior end of the animal. Frayed cilia may be seen on the margins of the preparation. Osmium tetroxide fixation.



FIGURES 3-4.

RESULTS

When properly prepared by osmium tetroxide fixation the material contains fragments of pellicle of various sizes (Figs. 1–3), the isolated oral apparatus (Figs. 5, 6) and formed bodies of the cytoplasm (mitochondria, macronuclei). The units of kinety structure generally remain attached to the pellicular fragments in osmium tetroxide-fixed material but they may be obtained in isolated form (Fig. 4) from formalin-fixed animals. Since the electron photomicrographs show interesting details of structure in the pellicle, kinety system and oral apparatus, these will be considered separately.

A. Structure of the pellicle

The pellicle is a thin membrane with three structurally distinct regions. These are most evident in Figure 1. This figure shows that the inner surface of the pellicle consists of (1) a "background area" of rather uniform granular appearance, (2) a "chain" of ringlike thickenings parallel to and midway between the kineties and (3) a finely striated area immediately to the right of each kinety.

Background area

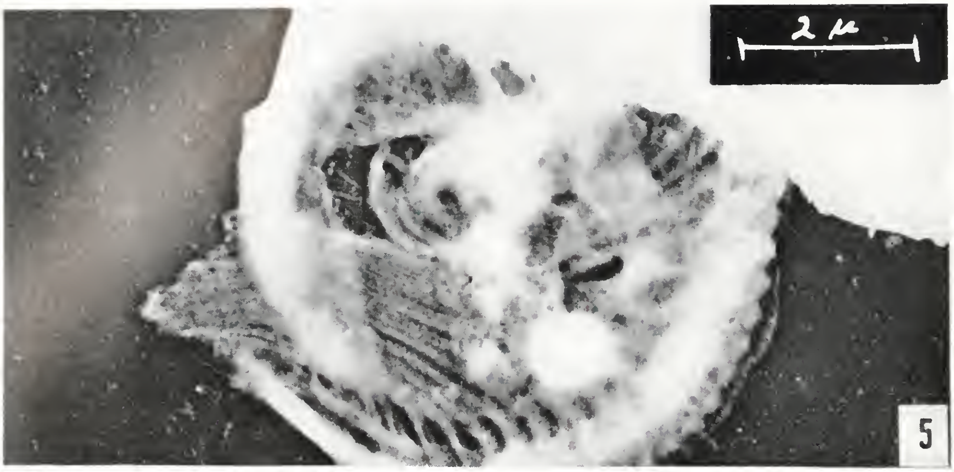
This region warrants little comment. The granular appearance of this material may be ascribed to macromolecular components of the inner surface of the membrane or to sub-pellicular protoplasmic components which have remained attached to the membrane proper. Study of this region at high magnifications and with special techniques might reveal further interesting details, possibly comparable to those of the erythrocyte membrane (Hillier and Hoffman, 1953).

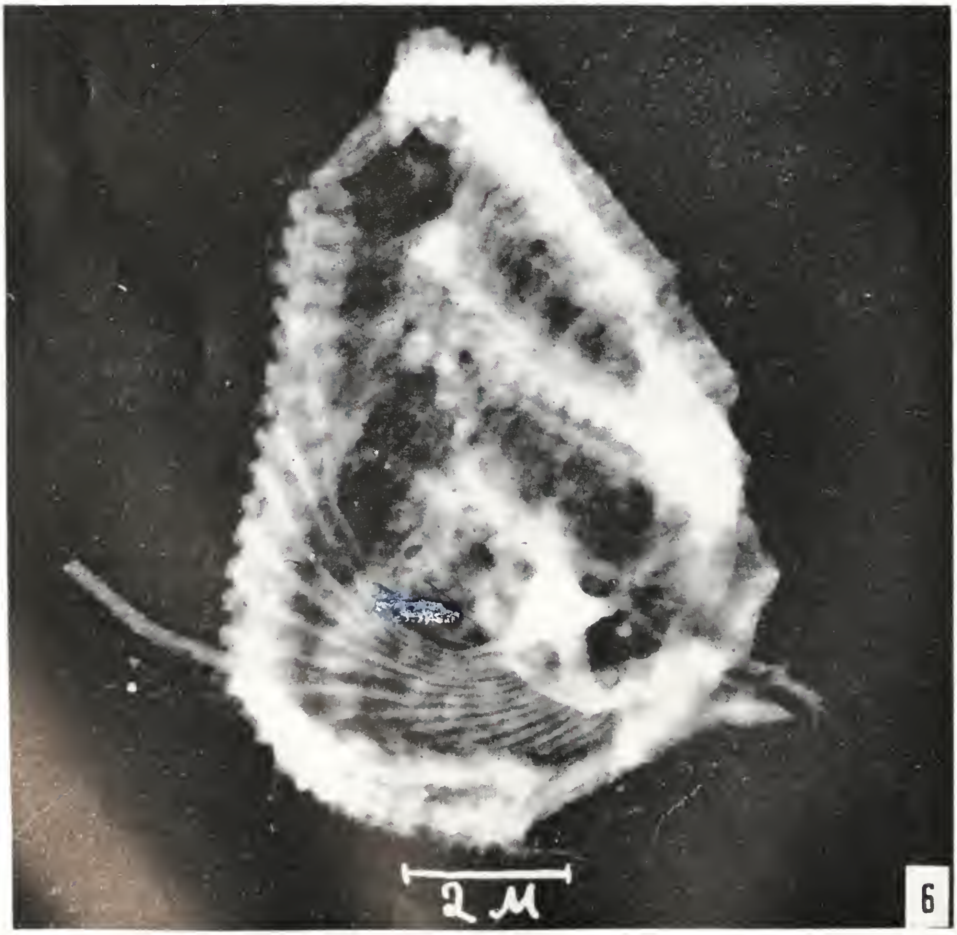
Chains of ring-like thickenings

The chains of ring-like thickenings are the most striking structural feature of the pellicle. Two systems of such chains appear to be present in *Tetrahymena.* The first and more prominent of these occurs with great regularity and consists of chains located midway between, and parallel to, the kineties. The second, less evident system parallels both the first system and the kineties and is located at the level of the kineties. This second system is found in many but not all preparations but it may occur generally, for this region of the pellicle is frequently obscured to a greater or less extent by the kinetosomes, kinetodesmal fibrils and cilia. The

FIGURE 3. "Simulated cross section." The pellicular fragment has folded upon itself. The fold forms the left margin of the specimen, the outer surfaces of the pellicle are opposed, with the frayed cilium from the central kinety unit sandwiched between these surfaces. The fold is oblique to the axis of the animal. Therefore the three prominent kinety units belong to three different kineties. The chains of ring-shaped thickenings run diagonally from lower right to upper left in the upper layer of the pellicle. They pass around the fold to the lower layer of pellicle and proceed from lower left to upper right, thereby forming an angle of approximately 90° with the parent row in the upper layer. The two systems of ring-shaped chains may be seen in this preparation. One of these is located at the level of the kineties, the other runs midway between kineties. Osmium tetroxide fixation.

FIGURE 4. The isolated unit of kinety structure. The long cilium consists of a narrow terminal portion, the main shaft with indications of longitudinal fibrils and the basal bulb. This bulb is to be distinguished from the kinetosome situated just below the bulb (compare with Fig. 3). The kinetodesmal fibrils which arise from the kinetosome again show periodicity. Formalin fixation.





FIGURES 5–6.

first system but not the second is evident in Figures 1 and 2. Both systems are present in Figure 3 (see caption to Fig. 3 for details).

Although the chains of ring-shaped thickenings occur with great regularity, their form is somewhat variable. This variability may be ascribed in part to variations in fixation. The rings are usually more prominent following prolonged fixation (osmium tetroxide) and their centers frequently appear more transparent in such preparations. Other variations can not be ascribed to fixation and may result in part from uneven stretching of these thin membranes during drying. In its simplest form the chain is a single even row of closely spaced rings (Fig. 2) but this construction, while not uncommon, is sometimes modified so that the chains consist of bands two or more rings wide (Fig. 1). In other preparations the chains may branch or the rings may be widely scattered in the granular area of the pellicle. However, these last variations are exceptional.

The striated area

The delicate striated areas are apparent only in favorable preparations but in these they occur regularly and are constant in position and form (Fig. 1). These areas are situated immediately to the right of each kinety.[5] Each consists of a narrow ($0.25\,\mu$ wide) band of material which runs parallel to the adjacent kinety and to the long axis of the animal. The characteristic feature of these bands is the collection of fine, closely spaced ridges (fibers?) that run lengthwise along the bands. The origin, composition and function of these bands are unknown. They are assumed to be part of the pellicle proper because of their constancy of occurrence and form. However, the possibility that they consist of sub-pellicular material adhering to the pellicle has not been excluded.

B. The kinety system

It will be recalled (Metz, Pitelka and Westfall, 1953) that the kineties of *Paramecium* are compound structures. They are built up of discrete units. Each of these units consists of a cilium, a kinestosome (ciliary basal body) and a relatively short, tapering, kinetodesmal fibril. These units are associated by their kinetodesmal fibrils. The fibrils overlap in shingle-like fashion to form a bundle, the kinetodesma (neuromotor or silver line fiber) of earlier light and electron microscope studies (Bretschneider, 1950; Sedar, 1952). The kineties of *Tetrahymena* are similar in structure to those of *Paramecium* in all important essentials.

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[5] "Right" as employed here refers to the animal's right. When the pellicle is viewed from within and the figure is oriented with the anterior end of the animal upward (Fig. 1), then the animal's right will correspond to the observer's right. When the fragment is viewed from the outside, with the anterior end upward, the animal's right will be the observer's left.

FIGURE 5. The posterior part of the oral apparatus viewed from its inner surface. The "fan" of parallel fibers is clearly seen to pass to the left and beyond the margin of the oral apparatus from the undulating membrane (membranelle I). At their origins the fibers appear to be in pairs or to have double origins in the lower part of the figure. Traces of the complex systems of fibers which interconnect the membranelles are visible in the upper part of the figure. Osmium tetroxide fixation.

FIGURE 6. The oral apparatus viewed from the outer surface. The base of the undulating or first membranelle passes along the right side (observer's left) of the preparation. A portion of one cilium remains attached to the base of this membranelle. The base of the second membranelle is located on the anterior left margin of the preparation. The third and fourth membranelle bases are located more posteriorly. The fan of fibers from the first membranelle passes to the left and under the left margin of the preparation. These fibers presumably pass down the wall of the cytopharynx in the intact animal. See text for further details. Osmium tetroxide fixation.

The unit of kinety structure

Each kinety of *Tetrahymena* is composed of discrete units. One such unit is shown in Figure 4. This isolated structure is composed of a cilium, a kinetosome and a kinetodesmal fibril. The cilium and kinetodesmal fibril form an angle of approximately ninety degrees as they arise from the kinetosome.

The *cilium* of *Tetrahymena* has the usual structure since it appears to consist of an outer limiting membrane (Fig. 4) enclosing a number of longitudinally arranged fibers. With mild fixation the membrane breaks down and the fibers fray out (Figs. 1, 2, 3). In the intact cilium (Fig. 4) three regions may be distinguished. These include 1) the main shaft, 2) a distal, narrow portion constituting $^1/_6$th of the cilium and 3) a proximal bulb. A similar narrow tip has been described in *Paramecium* (Metz, 1954) but the proximal bulb is evidently unique. This bulb is located at the base of the cilium as the latter structure passes through the pellicle. The proximal ciliary bulb should not be confused with the kinetosome. These two structures are separated by a constriction at the level of the pellicle. The relative positions of the ciliary bulb, pellicle and kinetosome may be determined by comparing Figures 3 and 4. Figure 3 shows a simulated cross section—actually a folded pellicular fragment with the outer pellicular surfaces opposed (see caption to Fig. 3 for details). One kinety unit is located on the margin of the fold and the pellicle is seen to pass between the proximal bulb of the cilium and the kinetosome. The ciliary bulb may be an artifact of preparation. However, its presence in both formalin and osmium tetroxide preparations does not favor this possibility.

The *kinetosome* appears to be a double structure (over-all dimensions 0.2 × 0.3 μ) consisting of two bodies separated by a constriction perpendicular to the surface in the transverse plane of the animal. One of these bodies, the primary one, appears to be slightly larger than the other and gives origin to both the cilium and the kinetodesmal fibril. The second body forms an appendage to the first, 180° from the site of origin of the kinetodesmal fibril. These relations are evident in Figure 3.

The *kinetodesmal fibrils* arise from the kinetosomes and run just below and parallel to the pellicle (Figs. 1, 2, 3). These structures gradually taper to a fine point from a base diameter of approximately 0.12 μ (calculated from Figs. 3 and 4; it is assumed that the specimens have suffered no appreciable flattening since they cast well proportioned shadows). Calculations from several preparations indicate a length of 1.5 to 2 microns for the fibrils. The average length of fibrils in Figure 1 is 1.5 μ; lengths in Figures 3 and 4 are 2.1 microns. The kinetodesmal fibrils show an interesting periodicity in favorable shadow-cast preparations (especially clear in Fig. 3). This periodic organization appears as a series of transverse ridges separated by constrictions in the preparations so far examined, but further study may resolve these into spiral ridges similar to those of the *Paramecium* kinetodesmal fibrils (Metz, Pitelka and Westfall, 1953).

Association of the units to form kineties

In *Tetrahymena* as in *Paramecium*, the units of kinety structure do not appear to exist as unrelated, randomly arranged, isolates. They are organized to form larger structural (and probably functional) units, the kineties. The units are arranged in longitudinal rows with the kinetosomes and cilia spaced at regular intervals (Figs. 1 and 2). The individual kinetodesmal fibrils are directed along these longitudinal rows and since they are longer than the distance between kinetosomes, they overlap in shingle-like fashion (Fig. 1). This overlap associates one kinety unit with the next adjacent unit. The series of overlapping fibrils constitutes the kinetodesma of the light microscopist. This construction is essentially the same as that found in *Paramecium*. In *Tetrahymena*, however, the kinetodesmal fibrils are so short (less than two interciliary distances) that true bundles of fibrils are never formed. A cross section of a kinety at any level should not show segments of more than two fibrils. In *Paramecium* the maximum number appears to be five.

In life the association of the fibrils in the region of overlap is presumably an intimate one and it appears to be so in some electron photomicrographs (Fig. 2), but in many preparations (Figs. 1, 3) the fibrils are widely separated at the region of overlap. This may be ascribed to the violent action of the sonic treatment, surface tension forces during drying and the relatively small area of contact between the fibrils. This failure to observe intimate association in most cases serves to emphasize the apparent absence of any sheath or cementing material binding the fibrils together. Indeed, no information is yet available concerning the details of this association. It is one of the more interesting problems arising from the study.

Polarity

Examination of the figures reveals that the kinetodesmal fibrils of the unit structures all extend in the same direction from their kinetosomes. They are polarized. Therefore, it becomes a matter of some interest to establish the direction of this polarity; to determine whether the fibrils extend toward the anterior or posterior end of the animal. Three independent observations bear upon this problem and they show that the fibrils pass toward the anterior end of the animal. These observations are the following: 1) The kinetodesmal fibrils pass to one side of the next adjacent kinetosome (Fig. 1). This arrangement is regular and consistent in all electron photomicrographs except for an occasional fibril. These exceptional cases may be ascribed to alterations of the pattern during preparation. Indeed it is astonishing that the pattern is so regular considering the violent nature of the treatment. This pattern is believed to be the basis for the "rule of desmodexy" (Chatton and Lwoff, 1935; Lwoff, 1950). This rule states that kinetosomes always lie to the left (animal's left) of the kinetodesma. When the photomicrographs are oriented according to desmodexy, the fibrils are found to pass forward, *i.e.*, anteriorly. This relation is illustrated in Figure 1. Since this figure shows the inner surface of the pellicular fragment, the observer's right and left correspond to the animal's right and left. 2) The isolated oral apparatus is highly asymmetrical (Fig. 6) and is easily oriented (see next section) with respect to the anterior-posterior axis of the animal. Occasionally the oral apparatus is isolated with pieces of adjacent pellicle attached and the orientation of the kinetodesmal fibrils attached to such pellicle can be established with respect to the anterior-posterior axis of the neighboring oral apparatus. Nine cases of this sort were examined. In eight of these the fibrils were directed anteriorly. In the 9th the fibrils were directed posteriorly but this may not be a valid case for it is possible that this figure represents an isolated oral apparatus lying over a fragment of unrelated pellicle. 3) When subjected to mild sonic treatment large segments of individual animals may be obtained. In favorable preparations these are largely free of internal protoplasm. Such "eviscerated" animals may retain the original form of the organism. The posterior end of such a preparation is illustrated in Figure 2. The fact that there are two layers of pellicle, one over the other, renders it difficult to follow some of the kineties and to determine which kinety belongs to which layer, but the figure clearly shows that the individual kinetodesmal fibrils pass anteriorly from their kinetosomes.

C. Oral anatomy

The preparations obtained by sonic dissection contain large numbers of oral structures in various degrees of isolation (see previous section under polarity). Representative examples are shown in Figures 5 and 6. With the exception of the oral cilia these preparations appear to consist of substantially the entire oral apparatus including the four "membranelles" and their interconnecting fiber systems.

The orientation of these isolates is readily established. This may be done by reference to the abundant figures in the literature from light microscope studies, by reversing argument number 2 presented above in the section on polarity, by examining large fragments (argument No. 3 in the above section on polarity) and by phase microscope examination of fixed material preliminary to sonic treatment. Applying these criteria the specimen illustrated in Figure 6 is found to be an outside view of the oral complex and the foundations of three of the four "membranelles" are readily identified. The right (animal's right, observer's left) and about half the posterior margin of the oral complex consist of the first of these, the so called "undulating membrane." A portion of one component cilium remains attached to the foundation of this membrane. The second "membranelle" is readily identified on the left (animal's left, observer's right) anterior margin and the third is located immediately posterior and nearly parallel to the second. The fourth "membranelle" is almost certainly situated in the electron-opaque material posterior to the third "membranelle," where a number of kinetosomes appear to be located. However, this region has not been observed clearly in the outside views of the oral apparatus and it is equally obscure in inside views. These complexes and their interconnecting fiber systems invite the detailed descriptions given below.

Membranelle No. 1 (undulating membrane)

The foundations of the membranelles are relatively dense and reveal less detail than might be desired. The first membranelle is no exception. It appears to consist of a marginal row of cilium bases, presumably kinetosomes. Segmentally arranged masses of material, 0.8 μ long, extend "medially" (presumably toward the interior of the animal) from these. They appear to correspond in number, but not necessarily in position to the ciliary bases and are readily identified in the anterior portion of the membranelle (Fig. 6). In some figures (Fig. 5) these masses appear to be broken up in a fashion to suggest at least three parallel rows of cilium bases. However, light microscope studies (*e.g.*, Furgason, 1940) show only one row of cilia in this membrane and electron microscope figures of specimens with intact cilia support this view. A suggestion of a longitudinal fiber (or series of short overlapping fibers?) is visible just medial to the cilium bases in the anterior end of the membranelle foundation (Fig. 6).

Although these details of structure have their interest, the most striking feature of the membranelle is the fan-shaped series of fibers that extend from it. In the figures this fan extends toward the left of the oral apparatus, under the left posterior margin and beyond. This is clearly shown in Figures 5 and 6. The fans of fibers illustrated in these figures are broken off relatively close to their origins. In some other figures, however, they appear to be relatively intact. In these they are found to extend at least 13 microns beyond the left margin of the oral apparatus. The fan of fibers tapers as it extends; whether this taper results from decrease in fiber number or from reduction in fiber diameter (or both) has not been determined, but if there is no reduction in number, the longest fibers must extend for approximately 20 microns (nearly half the length of the animal) from their bases. Presumably these fibers extend down or close to the right side of the gullet in the intact animal, but where they end has not been established.

The relation of these fibers to the kinetosomes of the membranelle cilia presents an interesting challenge to the microanatomist. The individual fibers of the fan arise at or near the kinetosomes of the membranelle. However, there appear to be fewer fibers than kinetosomes (16 and 28, respectively, in Fig. 6). No fibers are seen to extend to the first three or four kinetosomes in Figure 6, whereas more posteriorly a fiber seems to extend to every second kinetosome. The function of these fibers and their relation to other organelles present interesting problems for future study. The fact that some of the fibers appear double in Figure 5 enhances this interest.

Membranelles II, III and IV

The second and third membranelles each appear to consist of at least three rows of ciliary bases or kinetosomes. This is evident only for membranelle III in Figure 6. Figures not reproduced here show a similar condition in membranelle II. The region of membranelle IV has such high electron-scattering power that little detail is revealed in any of the photomicrographs so far obtained. A single row of kinetosomes does extend into this area from the anterior end of membranelle III and the individual kinetosomes of this row appear to be highly constant in position and number.

The four membranelles are interconnected by complex patterns of fibers that are revealed only in inside views of the oral apparatus. Prominent among these is a series of parallel fibers extending from membranelle II to membranelle III and what may be a continuation of this, a similar system extending from membrane III toward membranelle I (undulating membrane) and possibly connecting with the latter. These fibers appear to join with the membranelle kinetosomes. The appearance of these fibers suggests that they lie relatively deep below the oral pellicle. A second system of short, irregularly arranged fibers occupies a more superficial position. Neither of these systems has been studied in detail and they must be examined more thoroughly before an accurate description can be given.

Oral and kinetodesmal fibrils compared

A comparison of the oral fibers (Figs. 5, 6), especially the fan fibers from membranelle I, with the kinetodesmal fibrils (Figs. 1, 2, 3, 4) of the body ciliature immediately reveals that the two types of fibers are quite different in structure. The kinetodesmal fibrils are short and rather uniform in length whereas the oral fibers appear to vary in length from membranelle to membranelle. The fan fibers in particular are very long. Moreover, the construction of the fibers from the two regions is strikingly different. The kinetodesmal fibrils have the marked periodic organization, clearly revealed in Figure 3. No trace of this periodicity appears in any of the figures of the oral fibers. These figures include fibers that are shadowed along, and at right angles to, the fiber axis. Some figures include both kinetodesmal fibrils with clean-cut periodicity and oral fibers lacking such organization. If the oral and kinetodesmal fibers are homologous structures, as would appear to be the case considering the currently accepted view of stomatogenesis, then these differences in organization must reflect specific differences in a single morphogenetic machine. Since this machine must operate fairly close to the macromolecular level, it and its products warrant special attention and more exhaustive study.

Discussion

This study provides new information of two sorts. The first and more significant of these is confirmatory information of a general nature, the second concerns the specific, detailed morphology of *Tetrahymena.*

Confirmatory

The observations reported here confirm the major contribution of the first study in this series. This contribution concerns the structure of the kineties of ciliates. These are now found to be built up of discrete units in *Tetrohymena* as well as in *Paramecium.* Each kinety unit consists of a cilium, kinetosome and a short tapering kinetodesmal fibril. The units are associated by overlap of the kinetodesmal fibrils to form the kineties and they are polarized toward the anterior of the animal in this association. The confirmation of this basic structure in *Tetrahymena* lends some support to the view that this construction may be widespread if not universal among ciliates.

This basic kinety structure has certain interesting implications for the physiology of conduction and coordination and for morphogenesis in ciliates. These aspects have been discussed at some length in the first paper of this series (Metz, Pitelka and Westfall, 1953) and they need not be repeated in detail here. With regard to conduction it is sufficient to emphasize that an acetylcholine-acetylcholine esterase system appears to be involved in the locomotion of *Tetrahymena* (Seaman, 1951; Seaman and Houlihan, 1951). This may operate in "synaptic" transmission from the kinetodesmal fibril of one kinety unit to that of the next unit (Metz, Pitelka and Westfall, 1953). The operation of such a mechanism is more readily visualized in *Tetrahymena* than in *Paramecium* because of the relatively simpler construction in the former organism. In *Tetrahymena* the overlapping kinetodesmal fibrils are so short that any single fibril contacts only the fibrils of the two units immediately adjacent to it (one anterior, the other posterior, to the given unit). Consequently, any coordinating impulse would necessarily pass stepwise from one kinetodesmal fibril to the next adjacent fibril. In *Paramecium* the kinetodesmal fibrils are relatively much longer, so much longer that they form bundles up to five fibrils in cross section.

Orderly transmission here would seem to require some selective agency (Metz, Pitelka and Westfall, 1953).

Because of its relative simplicity the fibrillar system of *Tetrahymena* should be favorable material for morphogenetic studies at the electron microscope level. It should not be difficult to obtain division stages showing multiplication of the kinety units. Even stages in stomatogenesis are not beyond expectation. But until such preparations are obtained, discussion of morphogenesis beyond that already given (Metz, Pitelka and Westfall, 1953) is of no great value.

Specific morphology

The morphology of *Tetrahymena* and related organisms has been studied extensively by a number of investigators using light optics combined with silver staining. Furgason (1940) and Corliss (1952a, 1953) have reviewed this work thoroughly and their accounts will serve as the basis for the following discussion. The electron optical preparations clarify to some extent the confusion that exists regarding the detailed anatomy of the "ciliary meridians" and certain aspects of the oral morphology in *Tetrahymena*. These particular problems will be considered here.

The ciliary meridians, as revealed by light optics and silver impregnation, are complex and somewhat variable structures. The most constant features include the "primary meridians" connecting the ciliary bases in longitudinal rows and "secondary meridians" running parallel to and approximately midway between the primary meridians. In some preparations even a third or tertiary meridional structure is described. Aside from these meridians transverse structures are frequently observed which extend on the left from the primary meridian to the vicinity of the secondary meridian. The fact that the appearance of these structures varies depending upon the extent of silver impregnation, has led some investigators to the view that many of the described structures are artifacts (*e.g.*, von Gelei, 1935). These structures may be "artifacts" in the sense that they do not exist in the living animal as actual fibers; but there must certainly be some specific, physical basis for them.

With the detailed picture of the kineties and associated pellicle now available from the electron photomicrographs the physical basis for certain of the silver structures may be discussed with some confidence. The "primary meridians," at least in heavily impregnated specimens, lie in the region of the overlapping kinetodesmal fibrils. The silver probably precipitates around, over, or in these fibers to give the effect observed in heavily impregnated material. In lightly treated preparations the meridians assume a lateral loop effect to the left between adjacent kinetosomes. The known structure of the system does not readily account for this phenomenon; nor does it account for the lateral extensions from the primary meridians in heavily impregnated material. However, in over-fixed animals prepared for electron microscopy (actually quite lightly fixed), cytoplasmic material appears to precipitate preferentially over the kineties and to extend as lateral projections in a more or less regular fashion (see Methods section). It is possible that such material serves as sites for silver precipitation.

The chains of ring-like thickenings in the pellicle readily account for the "secondary meridians" of light microscopy, assuming that some silver precipitates in the region of these structures. The longitudinally striated area of the pellicle lies to the right of the kineties and apparently remains rather free of silver, although a fine "fiber" is sometimes observed in this region with light optics.

The above account offers explanations for most of the meridional structures described in silver impregnated material. However, it should be recalled that the subpellicular cytoplasm was removed by the method of preparation for electron microscopy so there is ample room for further speculation. Indeed there appear to be many possible explanations for some of the structures observed in silver preparations and it is unlikely that exhausting them here would serve a useful purpose.

The electron photomicrographs of the oral structures reveal the wealth of detail described earlier in this paper. The relation of this detail to the interpretation of light optical studies should be obvious. However, there are two points that deserve further mention.

One of these is the margin of the oral apparatus. This is delimited on the right and part of the posterior edge by the first or "undulating" membranelle and on the left anterior margin by the second membranelle. Elsewhere the margins are free of membranelle structure. In these free regions the marginal pellicle may be somewhat thickened, but it appears more likely that it is supported by the underlying fiber network. Indeed it is likely that the entire oral apparatus is held together in these fixed preparations by the complex, interconnecting fiber system. Whether or not this fiber system serves a supportive function in the living animal is problematical. Nothing suggesting the circumoral ring of light optics appeared in the electron photomicrographs.

The second point of particular interest concerns the broad fan of fibers associated with the first or undulating membranelle. This structure has not been revealed in detail by light optical studies, but the rods, ribs (Corliss, 1953) and "zone intermédiaire" (Fauré-Fremiet, 1948b) that appear in silver preparations certainly represent fragments of the fan fibers. Furthermore, there is no longer any excuse for confusing the individual fibers of the fan with cilia.

Favorable preparations of the pre-oral region have not yet been obtained. The inferior ones that have been examined fail to show the "intermeridional connecting fibers" of light microscopy. Undoubtedly some basis for these in the form of fibers or pellicular sculpturing as well as other interesting morphological details will reward further investigation.

The electron microscope at North Carolina State College was used in this study. We wish to thank Dr. A. C. Menius and Mr. W. T. Withers for its use and for their assistance.

We also wish to thank Dr. D. P. Costello and the members of his department for the hospitality of their laboratories, for their interest in the work and for many helpful suggestions.

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SUMMARY

1. Macronuclei, mitochondria, fragments of the pellicle and the oral apparatus of *Tetrahymena* may be isolated for electron microscopy by sonic dissection of osmium tetroxide fixed material. The fine morphology of the pellicle, the attached fibrillar structures and the oral apparatus are described.

2. The pellicle consists of a thin membrane which is sculptured to give a striated area immediately to the right of, and parallel to, each kinety, and two longitudinal ring-like thickenings, one at the level of and the other midway between the kineties.

3. The kineties of *Tetrahymena,* like those of *Paramecium,* are compound structures built up of discrete units. Each unit consists of three parts:

(a) The cilium which passes through a ring-shaped thickening in the pellicle.
(b) The ciliary basal body or kinetosome. This structure consists of two parts separated by a constriction.
(c) A short, tapering, kinetodesmal fibril which arises from the kinetosome.

These units of kinety structure are associated by their kinetodesmal fibrils. These fibrils overlap in shingle-like fashion to form the kinetodesma of the light microscopist. The individual kinetodesmal fibrils are highly polarized in this association. They all taper toward the anterior end of the animal. This mode of organization has now been demonstrated clearly in two ciliates, *Tetrahymena* and *Paramecium.* It will not be surprising if it is found to occur generally in ciliates.

4. The detailed structure of the oral apparatus, including the bases of the four membranelles and certain of their fibers, is revealed with new clarity. Prominent among the latter is a large fan-shaped group of fibers which arises from the first or undulating membranelle and probably extends deep into the interior of the animal.

5. The physical basis for some of the structures revealed to the light microscopist by silver impregnation is discussed.

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THE UPTAKE AND UTILIZATION OF PHOSPHATE IONS FROM SEA WATER BY THE AMERICAN OYSTER, CRASSOSTREA VIRGINICA (GMEL.)[1]

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Phosphorus is of physiological importance to mollusks not only in their carbohydrate metabolism and energy transfer systems but also in shell deposition. Both Manigault (1939) and Bevelander and Benzer (1948) have associated shell deposition with phosphatase activity, and the latter also suggested that the first material to be deposited is calcium phosphate, which is changed into calcium carbonate in the presence of a phosphatase. Love and Frommhagen (1953) have found further evidence that calcium phosphate is the precursor of calcium carbonate in *Mactra* (*Spisula*) *solidissima*. Bevelander (1952) exposed several species of mollusks to $P^{32}O_4$ ions in sea water and found in radioautographs that P^{32} was localized on the inner surface of the mantle, below the surface epithelium. Since aqueous methods were used in the preparation of the tissues, the P^{32} shown by his radioautographs was only that fraction which had entered water-insoluble combinations.

Two sources of phosphorus are potentially available to marine, ciliary feeding mollusks: that combined in suspended particulate matter, such as plankton and detritus, and that in solution in sea water in various chemical combinations. The latter may be divided into phosphate ions in equilibrium with various cations and dissolved organic compounds which include phosphorus in their makeup.

Ronkin (1950) has shown that $P^{32}O_4$ ions are absorbed by the excised gill of *Mytilus edulis,* and he suggests that some of this phosphorus is used in the production of adenosine triphosphate by the gill, to be used in maintaining the extensive ciliary action there. It has been further shown (Pomeroy and Haskin, 1951) that phosphate ions are absorbed by the oyster from sea water principally through the gills, and that these ions appear rapidly in the blood and eventually in other tissues. This report gives further information on the uptake and distribution of phosphorus in the oyster, the absorptive mechanism, and the importance of the direct utilization of ions from the surrounding water.

MATERIALS AND METHODS

Phosphorus analyses on sea water were performed by the method of Redfield *et al.* (1937), modified for the use of a photometer. Phosphorus analyses on tissues were made by wet-ashing them in sulfuric and nitric acid, driving off the excess nitric acid by slow heating, carbonization of the organic matter in the remaining sulfuric acid, oxidation with 30 per cent hydrogen peroxide, and proceeding as with sea water samples. All tissues were collected and ashed in duplicate. Aliquots from the wet-ashed tissue samples were evaporated to dryness on planchets of uniform area and counts made of their radioactivity using a scaler and counting tube with an end-window density of 1.4 mg./cm.^{2}. Samples were sufficiently small and dilute to prevent appreciable self-absorption. This was verified by counts of a suitable graduated series of sample dilutions.

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Radioautographs were made by two of the methods of Holt and Warren (1950), one of which involved quick freezing and vacuum dehydration while the other involved fixation in neutral alcohol-formalin fixative. In addition, radioautographs were prepared of frozen gross serial sections of whole oysters. The latter were particularly useful in studying phosphorus distribution. Their preparation involved quick-freezing the whole oyster, rapidly preparing gross sections three to four millimeters in thickness of the frozen specimen, and clamping them against cold nuclear track plates. The sections were kept frozen throughout the cutting, clamping, and exposure periods. Eastman NTB nuclear track plates with a ten-micron emulsion proved satisfactory, even when exposure involved prolonged storage in a freezer. The simplicity of the process permitted the preparation of radioautographs of serial gross sections of a number of oysters with a minimum of time and equipment. Since the sections remained frozen throughout, there was no loss or movement of soluble radioisotopes.

The oysters used were Delaware Bay market-size oysters. Nearly all the oysters were from a single lot which was stored in trays in the Bay. Small groups were brought to the laboratory by car periodically. There they were kept in a cold room, if not needed immediately. When stored in a cold room, they were placed in sea water at 20° C. for 24 hours before use, and were then transferred to fresh sea water. The responses of stored oysters were normal when compared with freshly collected ones.

All oysters were scrubbed with a stiff brush and washed in running water to remove sand, loose shell fragments, and attached organisms. Measurements of the radioactivity of the outer surface of oyster shells at the end of experiments indicated that there was little adsorption of phosphate ions or accumulation of radioactive phosphorus by such microflora as might have remained attached.

Each of the experiments described represents one of several replications made at somewhat varied initial phosphate concentrations.

OBSERVATIONS

1. *Uptake of phosphate by oysters*

Groups of six oysters were placed in individual battery jars, each with one liter of water containing 40 micrograms of phosphate phosphorus, to which 20 microcuries of P^{32} as H_3PO_4 had been added. The addition of the radioisotope did not increase the concentration of phosphate ions in the sea water significantly. A constant temperature of 18° C. was maintained. After various time intervals a group of six was removed for assay of the tissues. Duplicate samples were taken of several tissues. All oysters were observed to establish the fact that they were open while in the labelled sea water. The results are shown in Figure 1. The apparent drop in the mean specific activity of most tissues at 64 hours as compared with 16 hours is not significant. Individual variation was so great that uptake rates are only approximated by this method. This is particularly true in the case of the digestive gland, with a very low and variable uptake rate. It appears, however, that a steady state is reached at about 16 hours.

Several oysters which did not open while in the labelled sea water were also assayed. None showed any radioactivity in the tissues, although in most cases the pallial border was exposed, for the shells had been chipped to facilitate opening them when radioactive.

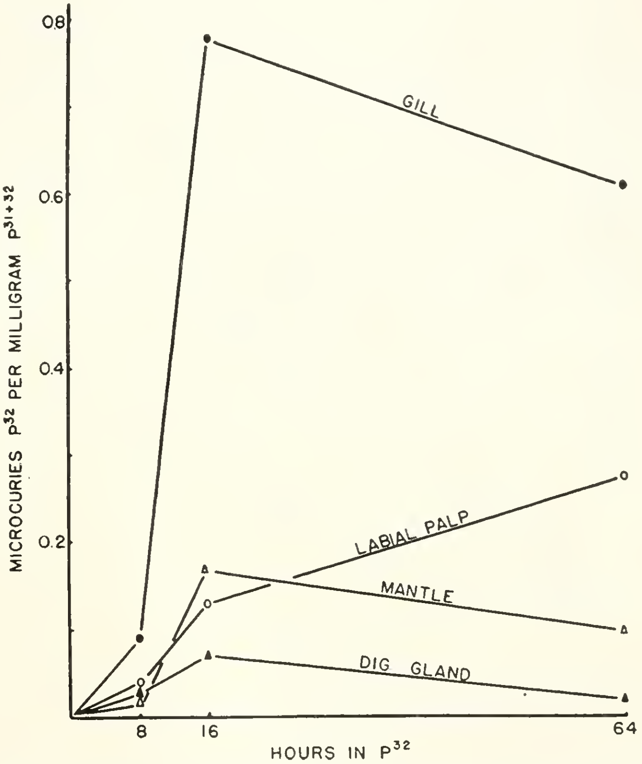

FIGURE 1. Uptake of phosphate ions labelled with P^{32} from sea water by groups of oysters. See text for description.

2. *Uptake of phosphate ions by excised oyster tissues*

Excised tissues from three oysters were placed in P^{32}-labelled sea water which was aerated and maintained at 18° C. in a water bath. The pieces of tissue were about one cm.^{2} in size. The amount of exposed cut surface varied, being comparatively little in the case of gills and great in the digestive gland and adductor muscle. After time intervals comparable to those in the preceding study, duplicate samples

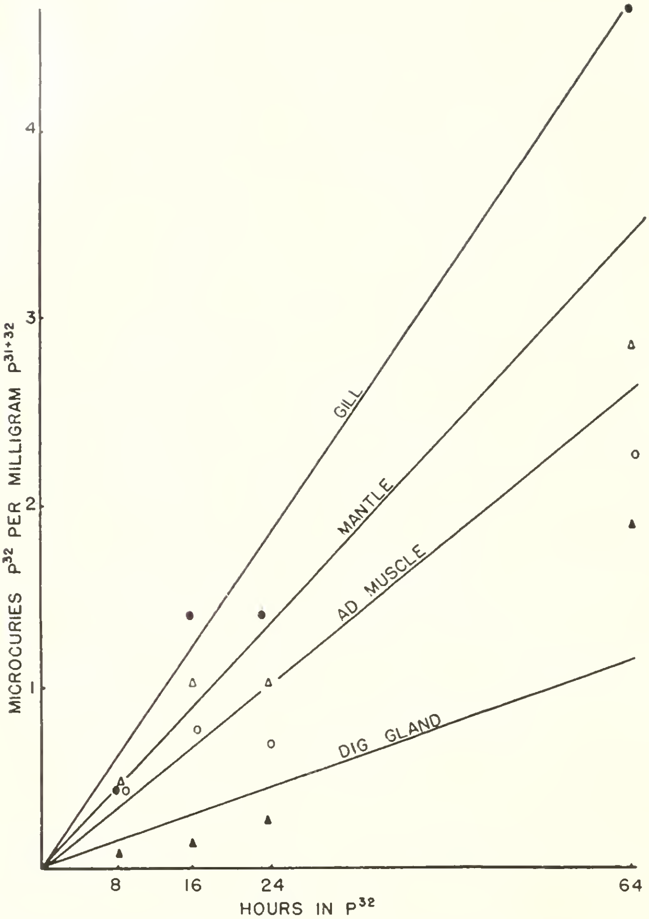

FIGURE 2. Uptake of phosphate ions labelled with P^{32} from sea water by groups of excised tissues of oysters. Gill, ●; mantle, △; adductor muscle, ○; digestive gland, ▲. See text for description.

of each tissue were removed and assayed. Figure 2 shows the results, with computed lines of best fit. The relationship of uptake to time is essentially linear.

3. *Labelled phosphorus in the blood*

Oysters were placed in labelled sea water as in the first experiment. After various time intervals samples of blood were removed from the heart and assayed. The results are shown in Table I. Each value is the mean of duplicate samples of six oysters. The total blood phosphorus varied in different individuals from eight to 30 micrograms per milliliter. However, there was a labile fraction which was relatively consistent in terms of per cent of total phosphorus.

TABLE I

Mean uptake of phosphate ions by the blood of oysters in sea water labelled with P^{32} at 18° C., expressed as per cent of total blood phosphorus

Hours in P^{32}	1	2	4	8	12
% of blood phosphorus labelled	0.03	0.05	0.08	0.08	0.09

4. *Excretion of phosphorus by oysters*

Several oysters were placed in individual aerated battery jars containing filtered sea water maintained at 18° C. The water was replaced at two- to four-day intervals for 33 days. Phosphorus determinations were made before and after the water was used. The average daily phosphorus excretion is shown in Table II. Each entry represents the mean daily value for the days the water was in use. The oysters had been in dry cold storage prior to the experiment, and probably voided some accumulated wastes at the beginning. After a week there was little change in the rate of phosphorus excretion. The oysters received no food other than the dissolved or exceedingly small materials which passed through the cotton filter.

TABLE II

Excretion of phosphorus by three oysters, expressed in micrograms of phosphorus excreted per 24 hours

Days:	2	5	7	10	12	16	20	26	33
No. 1	620	100	30	50	40	40	40	20	20
No. 2	560	110	100	80	50	40	40	20	20
No. 3	500	130	100	50	50	40	30	20	20

5. *Radioautographs of phosphorus distribution*

Oysters were placed in labelled sea water as in the first experiment, after which radioautographs were prepared of several tissues and of gross serial sections of whole oysters. After four hours in P^{32}-labelled sea water the gills showed P^{32} to be generally distributed in them. Other tissues showed no activity, except one specimen which showed activity in one of the peripheral blood vessels of the mantle. After 12 hours in P^{32} most tissues produced radioautographs of nearly uniform density. After 24 hours radioautographs were still uniform though more intensely darkened.

DISCUSSION AND CONCLUSIONS

After periods of one to four hours of exposure to $P^{32}O_4$-labelled sea water, P^{32} is detected in oysters only in the gills and blood taken from the heart, indicating that most, if not all, phosphate ions absorbed directly from sea water enter through the gills and reach other tissues via the blood. After 12 hours only about 0.1 per cent of the total blood phosphorus is derived directly from dissolved phosphate ions.

This small labile fraction may be one of the principal limitations of uptake rate for the organism as a whole. However, additional factors may limit the rate of uptake of phosphate ions by individual tissues. In repeated experiments with excised tissues, their respective uptake rates always fell in the same order. This suggests that each has a characteristic relative uptake rate.

The observation that radioautographs of the tissues of oysters which had been in sea water containing $P^{32}O_4$ ions were uniformly darkened suggests to us that most of the phosphate ions remain in the tissue fluids for the first 24 hours after absorption. At least, there is not enough concentration of the labelled ions in any tissue to cause a noticeably greater darkening of any portion of the radioautographs. The radioautographs of Bevelander (1952), however, show that in some mollusks a fraction is incorporated into insoluble materials within this period. If this is also true in the case of oysters, and probably it is, then the amount of absorbed phosphorus converted to water-insoluble compounds in 24 hours must represent a small fraction of the total absorbed phosphorus.

If in experiment (1) we take 0.1 microcurie per milligram of phosphorus as the approximate mean specific activity of the whole oyster after 24 hours in labelled sea water and assume (on the basis of many analyses) that the average oyster weighs 10 grams and contains 20 milligrams of phosphorus, then the oysters in this experiment absorbed four micrograms of phosphorus in 24 hours. The basal phosphorus loss through excretion is 20 micrograms in 24 hours. The amount of phosphorus obtained from ions in sea water in this case is about 20 per cent of the basic requirement of the oyster. However, in other experiments with different groups of oysters the fraction of the basal requirement obtained from ions in sea water varied from five to 50 per cent. These values must be considered to be only approximations.

The levelling-off of specific activity in the intact tissues, as contrasted with the continued accumulation in excised tissues, suggests that in the former the uptake is balanced by losses from the tissues. The physiological significance of this steady-state cannot be judged from available information.

Leenhardt's (1926) studies of *Gryphaea* (*Crassostrea*) *angulata* indicate that the gill circulation is by-passed by the principal blood vessels, but that there is a small exchange at all times between the gill circulation and the general circulation. If this is also true of *C. virginica*, it may be one factor limiting the utilization of dissolved phosphate. However, the gills, which obtain phosphate ions from the water without mediation by the blood, also reach a steady-state of uptake after about 16 hours. Therefore, some limiting factor other than the rate of circulation and the amount of phosphorus carried by the blood would seem to exist.

Since the gills take up relatively greater amounts of phosphate from the water and obtain it directly, it is interesting to speculate that the gills may be autonomous to some degree. They may obtain a substantial part of their nutritional needs directly by absorption and local phagocytosis.

The observation that no P^{32} entered oysters which remained closed during their exposure to labelled sea water, even when the margins of their shells were imperfect, shows that the pallial curtains are capable of completely sealing off the mantle cavity from the external medium. It further verifies the observation that the tissues of the pallial curtain do not play a large role in the direct absorption of phosphate ions from sea water.

A high degree of physiological variability was noted in the oysters used in this work, although every effort was made to use oysters from a single local population and of one size class for any given experiment. Great variability was also found by Wilbur and Jodrey (1952) in the shell deposition process. Space limitations and the large volumes of radioactive sea water involved made the use of larger groups of oysters impractical.

It is probable that other dissolved materials are absorbed through the pathway which has been demonstrated for phosphate ions. Preliminary studies with Ca^{45} indicate that relatively greater amounts of calcium are absorbed by the gills and carried in the blood. This may be the principal source of calcium for shell deposition.

SUMMARY

1. Phosphate ions in sea water are absorbed by oysters, principally by the gill, and are carried by the blood to all parts of the organism.

2. Directly absorbed phosphate ions are incorporated into the tissues of adult oysters slowly, most remaining in water-soluble forms 24 hours after absorption.

3. Significant amounts of phosphorus may be obtained by oysters through the direct absorption of phosphate ions from sea water.

4. After 12 hours in $P^{32}O_4$, directly absorbed phosphate ions make up less than 0.1 per cent of the total blood phosphorus. This may be one of the several factors which limit the utilization of phosphorus from sea water.

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THE ROLE OF SPECIFIC SURFACE ANTIGENS IN CELL ADHESION. PART I. THE REAGGREGATION OF SPONGE CELLS[1]

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In the "auto-antibody concept" of Tyler (1940, 1942, 1946, 1947) and the "molecular ecology" of Weiss (1941, 1947, 1950) one important part is the hypothesis that contiguous cell surfaces are normally held together, at least partly, by forces like those between antigens and homologous antibodies. The forces are assumed to be associated with specific macromolecules, of at least two stereochemically reciprocal types per cell, so held in the cell surface that they can combine from cell to cell. The hypothesis has been shown to be compatible with a diverse array of observational and experimental data, particularly by Tyler (1947) and Weiss (1947). However, both workers have emphasized the need for new experiments. "It is obviously essential for us first to obtain further evidence for or against the existence of such antigen-antibody like systems of complementary substances within cells" (Tyler, 1947; p. 17). "The subtle means by which cells can recognize each other and their appropriate environments are still wholly conjectural" (Weiss, 1950; p. 184).

It has been proposed (Tyler, 1947) that for further investigations along this line, the phenomena of selective reaggregation of dissociated sponges (Wilson, 1910; Galtsoff, 1925) and of the segregation of cells that occurs in certain combinations of embryonic tissues (Holtfreter, 1939) should constitute favorable material.

One type of experiment is suggested by the proposal that *macromolecules* are involved. Such molecules might act as antigens when injected from one species into another. Antisera so produced should affect, in a predictable way, processes involved in cell adhesion. Experiments of this type are reported here on dissociated *Microciona prolifera* and *Cliona celata*. In a second paper (Spiegel, 1954) experiments on embryonic cells of *Rana pipiens* and *Triton alpestris* are reported.

NORMAL REAGGREGATION AND SEGREGATION

A review of the essential phenomena in sponge reaggregation and segregation may be a useful preliminary to consideration of the experimental results.

Wilson (1907) first showed that a sponge could be dissociated, by pressing through bolting cloth, into isolated cells which would form a number of reaggregates within a day's time. Galtsoff (1925) showed that clusters of about 2,000 or more cells are capable of reorganizing, after 5–6 days under proper conditions, into perfect miniature sponges. Various aspects of the phenomena have been studied by Wilson (1910, 1932), Galtsoff (1923, 1925, 1926, 1929), de Laubenfels (1927, 1932) and Brøndsted (1936) among others, and the following account is based partly on their observations and those of the present author.

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[1] Submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy.

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After disaggregation the cells slowly settle down. Upon coming in contact with the substratum, archeocytes and pinacocytes flatten out, adhere and put forth hyaline pseudopodia. They begin to move almost immediately. The archeocytes are more active and move at about 0.6–3.5 microns per minute, changing direction apparently at random roughly three times per hour. Under extremely favorable conditions their velocity may reach 20 microns per minute (Galtsoff, 1925). Movement continues for about 24 hours.

Upon coming in contact with one another, the cells generally coalesce. The plasma membranes of the apposed cells seem to join together zipper-wise, proceeding from the area of first contact. After 5–10 minutes, the surface of union between the two cells is indistinct or has vanished and it appears that the clear outer hyaloplasm has formed a common matrix for the two inner granuloplasms which remain distinct and separate from one another.

As new contacts continue to occur, larger and larger aggregates are formed, the number and size depending on the density of the original suspension and on temperature, osmotic pressure, ionic composition, pH of the medium, etc. Aggregates increase in size by addition of single cells or by coalescence with other aggregates. Under Galtsoff's standard conditions some aggregates were about 0.5 mm. in diameter, consisting of roughly 2000 cells, after one day. Neither number nor size of aggregates changes further after ameboid movement stops at the end of 24 hours.

Present observations reveal certain additional features of interest. *Movements of granules during cell coalescence.* In Microciona while a pair of cells is coalescing, their granules undergo a series of intense movements and shifts in position, resulting in a striking reorganization of the granuloplasm. The granules which seem originally to be distributed at random in the cytoplasm of an isolated cell reorient so as to lie immediately inside the surface of the granuloplasm. The archeocyte nuclei, which previously had been partly or wholly obscured by the granules, are now quite evident with conspicuous light green nucleoli. *Fusion of hyaloplasm in cell coalescence.* An aggregate of two cells is able to put out a pseudopodium about 15 microns long which is approximately twice that formed by an isolated cell. An aggregate of three cells may form pseudopodia ranging from 30 to 35 microns in length. Large aggregates of 100–200 cells occasionally put out pseudopodia 200–300 microns long, of quite striking appearance. In general pseudopodium size varies with about the 1.2 power of cell number. The hyaloplasm of all the cells evidently acts in some sense as a unit, freely available to the aggregate's single pseudopodium. The microscopic appearance during coalescence suggests that the interface between the hyaloplasms of two coalescing cells disappears.

EFFECTS OF ANTISERA ON REAGGREGATION AND SEGREGATION

Materials and Methods

Specimens of *Microciona prolifera* (the large encrusting red sponge) and *Cliona celata* (the yellow sulfur sponge) were employed. The colonies were kept in running sea water in the experiments done at the Marine Biological Laboratory. For work at the University of Rochester, living colonies were shipped from Woods Hole by Air Express and kept in sea water, replaced at two-day intervals, at a temperature of 1–2° C. Experimental results were similar under the two maintenance methods.

Quantitative studies of reaggregation. For the experiments, it seemed desirable to have a quantitative expression of the extent of reaggregation at successive times. The following method was used.

For each series of observations a fragment of fresh sponge was carefully dried with filter paper and a one-gram portion squeezed through bolting cloth into 40 ml. of sea water. Ten ml. of the suspension were transferred to a syracuse dish and examined under the microscope at 430 × magnification. At intervals the aggregates were counted in each of five microscope fields taken at random, and the values were averaged. For these counts "aggregate" was defined as any coalesced group of *four or more cells*, and it is in this sense that the word will be used hereafter. Four cells constitute the smallest grouping that can be readily recognized as an aggregate as distinct from cells in contact, in routine examinations.

Typical results over a 24-hour period are shown in Figure 1. Such a figure will be called a reaggregation curve and each of its ordinates, an aggregate count.

The curve is satisfactorily reproducible under standard conditions; as an example, seven unselected curves are shown in Figure 2. Up to four hours the extreme variation of counts at any one time is about two aggregates. Between four and eight hours counts are somewhat more variable. But they are quite uniform again at 24 hours, at least under normal conditions: 12 such counts, unselected, range from 3.9 to 4.5. The progress of reaggregation as illustrated by these curves

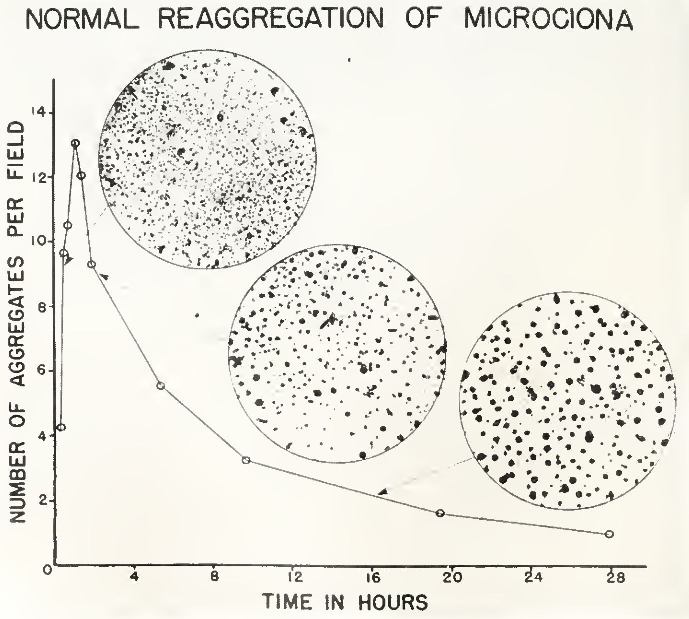

FIGURE 1. Reaggregation of *Microciona prolifera* in sea water.

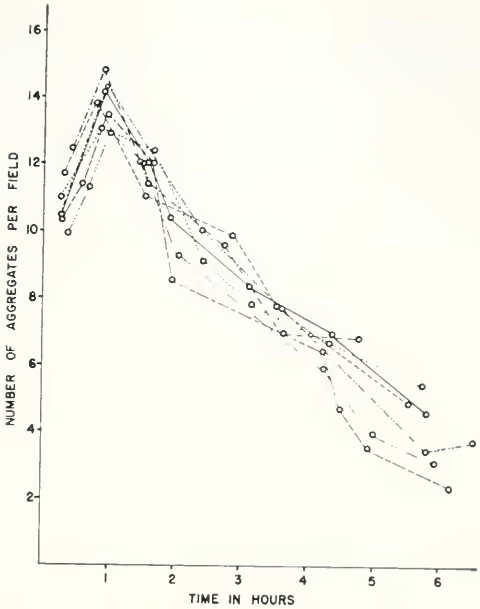

FIGURE 2. Reaggregation of *Microciona prolifera* in normal serum; seven unselected curves demonstrating reproducibility under standard conditions. Essentially the same curve is obtained in sea water.

shows an initial increase in number of aggregates (of small size) to a maximum at about one hour, followed by a decrease in number (fusion of aggregates with corresponding increase in size).

In a mixed suspension of the red-colored Microciona and the yellow Cliona cells, 24 hours after dispersal, the aggregates are observed to be species-specific, as reported by Wilson (1910), Galtsoff (1925) and de Laubenfels (1927). A few cells of one type in an aggregate prevailingly of the other species cannot readily be distinguished. If such admixture occurs, its extent is too slight to be readily detectable.

The Microciona aggregates formed in a mixed suspension are more numerous and correspondingly smaller than in a pure Microciona suspension. They are, however, normally well rounded with a well-defined hyaline membrane. Similarly, the Cliona aggregates, more and smaller than in pure Cliona preparations, have the characteristically jagged contour but easily seen hyaline coat of pure Cliona suspensions. Occasionally an aggregate of Microciona and one of Cliona are observed in close contact, but their hyaloplasms remain separate and the two fail to adhere.

It is important, for interpreting the experimental results, to note here that the condition seen at 24 hours is preceded by an initial temporary intermixture of the two types of cells. But at 4–8 hours, after dissociation, in most aggregates islands of cells of one species are seen in a matrix of the other species; they are apparently separating from it.

The smaller size of aggregates in mixed suspensions is perhaps partly a consequence of the sorting out: contacts which would lead to a permanent increase of aggregate size in a pure suspension are cancelled out in a mixed preparation by the later sorting out where interspecific contacts had occurred. To some extent each species may also interfere just mechanically with the other's aggregation as Galtsoff (1925) has shown glass particles and starch grains to do.

Preparation of stock suspensions. For each of these a piece of fresh sponge was cut into fragments 2–3 mm. across, which were carefully dried on filter paper. A known weight of fragments was squeezed through a small bag made of bolting silk, mesh 20, into sea water. Six types of suspensions were used, and freshly prepared before each use; they were as follows, M representing Microciona, and C, Cliona:

Stock suspension	1	2	3	4	5	6
Sponge (g.)	6.2M	6.2C	6.2M 6.2C	1M	6.2C	1 M 6.2C
Sea water (ml.)	20	20	40	40	40	40

Suspensions 1, 2 and 3 were used in preparations of antisera; their composition was so chosen as to give the same total nitrogen per ml.; 1.3 mg. in each, by micro-Kjeldahl determination. Suspensions 4, 5 and 6 were used in tests of antiserum effects; their composition was so chosen as to give about equal initial numbers of free cells per microscope field in 4 and 5.

Preparation of antisera. For most of the experiments the antisera were derived from nine New Zealand giant rabbits, three for each suspension. Nine subcutaneous injections of 0.1 ml. of stock suspension, were given over a period of three weeks.

At 8 days after the last injection blood was collected in sterile 50-ml. Pyrex centrifuge tubes, allowed to clot at room temperature, kept at 40° C. for one hour, the clot loosened, and the blood centrifuged at 350 G for 15 minutes. The clear straw-colored serum was pipetted off and the following were added per 50 ml. serum: 0.2 mg. Penicillin G potassium, 0.2 mg. Streptomycin calcium chloride complex and 0.2 mg. Aureomycin. The mixture was kept in sterile serum bottles at 1° C. until use.

Serum obtained from animals injected with Microciona cells will be referred to hereafter as anti-Microciona serum; others will be similarly called anti-Cliona and anti-Microciona : Cliona.

Test of antiserum titers. These were done to determine, by standard methods, that the sera actually contained antibodies to sponge antigens. A 6.2-gram portion of Microciona was homogenized for five minutes in a Ten Broeck glass homogenizer with 20 ml. of 0.01 *M* phosphate buffer at pH 7.2 and containing 0.85% NaCl. The homogenate was centrifuged at 485 G for 20 minutes and the supernatant collected. The same procedure was carried out with 6.2 grams of Cliona cells in 20 ml. of buffered saline and with 6.2 grams Microciona plus 6.2 grams Cliona in 40 ml. of buffered saline.

Precipitation tests were set up by layering 0.5 ml. of undiluted extract and of five 10-fold dilutions (1:10 to 1:100,000 in buffered saline) over 0.5 ml. of homologous, undiluted antiserum, or over saline or normal serum as controls in 10 × 75 mm. serological tubes. All tubes were incubated at 37° C. for one hour and then examined for presence of a precipitate ring at the interface. All nine sera produced rings of antigen-antibody complex at all antigen dilutions down through 1:10,000. Controls were negative. The tests show, then, the presence of antibodies to sponge antigens extractible in saline, although these do not necessarily mean that cell surface antigens are involved.

Tests of antiserum effects on reaggregation. Three ml. of an antiserum were mixed with an equal volume of double-strength van't Hoff solution alpha (58.44 grams NaCl, 1.62 grams KCl, 2.24 grams $CaCl_2$, 16.18 grams $MgCl_2.6H_2O$, 9.38 grams $MgSO_4.7H_2O$, distilled water to one liter mark), producing a medium approximately isosmotic with sea water. Four ml. of suspension 4, 5 or 6, freshly prepared, were added. Controls were 4-ml. portions of the same cell suspension added to three ml. of double-strength van't Hoff solution plus three ml. of serum from a normal uninjected rabbit (18 experiments), or to six ml. of ordinary strength van't Hoff (four experiments). In five experiments there were no simultaneous controls.

Altogether 63 antiserum and 22 control preparations were observed in 27 experiments; normal reaggregation was also followed in 13 other preparations, 10 in normal serum and three in van't Hoff solution.

RESULTS

Microciona in anti-Microciona serum

Reaggregation curves for Microciona cells during the first three hours in normal serum and in two anti-Microciona sera are shown in Figure 3 with photomicrographs at 2.75 hours. In two other antiserum preparations studied by reaggregation curves, results were obtained scarcely distinguishable from the two pictured here.

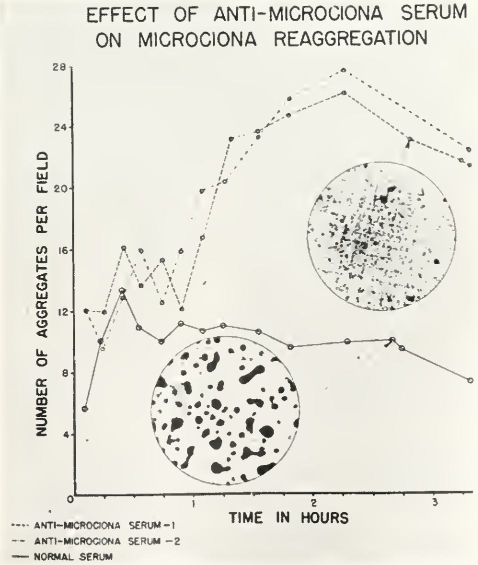

FIGURE 3. Effect of anti-Microciona serum on Microciona reaggregation.

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

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[1] [illegible]

[2] [illegible]

3. contact of some "cells" with the substratum, and adhesion, during preliminary swirling;

4. settling of initially suspended "cells" throughout the first 2 hours;

5. contact and coalescence among cells and aggregates creeping on the substratum.

A reaggregation curve during the first few hours reflects these processes jointly. But 1, 3, and 4 are really irrelevant to the phenomena of primary interest here. Furthermore the Microciona experiments in anti-Microciona serum showed that processes 2 and 5 operate alike, so that no significant information is obtained within the first few hours that could not be more easily gotten from any single later observation. Since the same would presumably be true for Cliona and for other antisera, attention was confined in subsequent experiments to observations at 24 hours after dissociation (when cell movement ceases and no further coalescence occurs).

Other combinations of sponge and antiserum

Microciona (Fig. 4). In anti-Cliona serum there were no signs that reaggregation had been inhibited. The aggregates in anti-Cliona serum were as large or larger than normal (Fig. 4). Five experiments gave consistent results, the aggregates counts at 24 hours averaging about 4.3.

In anti-Microciona:Cliona serum, Microciona formed aggregates of number and size intermediate between those in normal serum and in anti-Microciona serum; reaggregation was partly inhibited.

Cliona. There were more, and smaller, aggregates in anti-Cliona serum than in normal serum, indicating an inhibition of reaggregation analogous to that of Microciona in anti-Microciona serum.

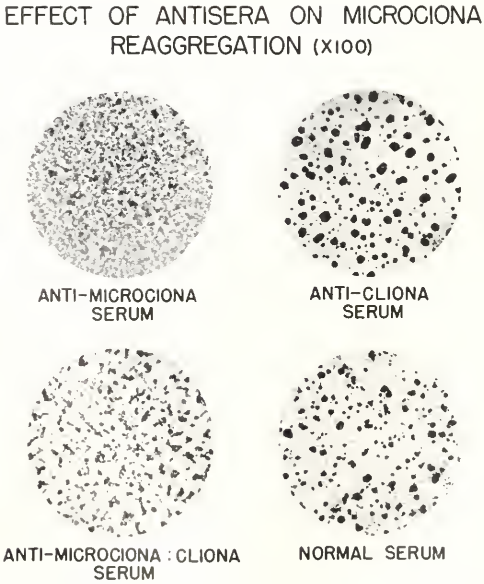


FIGURE 4. Reaggregation of Microciona in antisera.

In anti-Microciona:Cliona serum, there were fewer aggregates than in anti-Cliona serum but more, and smaller, than in normal serum. The inhibition was partial like that of Microciona in the same serum.

With anti-Microciona serum, there were fewer and larger aggregates than in anti-Cliona serum but smaller than in normal serum, and scarcely larger than in anti-Microciona:Cliona serum.

Microciona-Cliona mixtures. In normal serum the initially mixed cells sorted out. The Microciona aggregates were smaller than are formed by pure Microciona as were also the Cliona aggregates. The probable reason for this has already been suggested above and has nothing to do with their surface antigens.

In anti-Cliona, as in normal, serum, there were no mixed aggregates and the Microciona aggregates were normal, though smaller than formed by Microciona alone in normal or in anti-Cliona serum. The Cliona components of the suspension were much like Cliona in anti-Cliona. The results are what would be expected if anti-Cliona serum contained antibodies to surface antigens of Cliona but not Microciona.

In anti-Microciona serum, the mixed suspension did not form any large aggregates of the Microciona type, but many isolated cells and small clusters were seen. The larger aggregates were found to consist of Cliona cells. Most of them were about as large as formed by Cliona alone in anti-Microciona serum. They were smaller, though, than those of Cliona alone in normal serum. This can be interpreted as a consequence of the cancelling of previous associations by ejections of Microciona cells from mixed clusters as suggested above for mixed cells in normal serum.

In anti-Microciona:Cliona serum, a result was obtained diametrically opposite to the effect of the other antisera on pure or mixed suspensions. There were large aggregates of general shape intermediate between Microciona in normal serum and Cliona in normal serum. Many of the aggregates were much larger than in any other cell-serum combination. At the time of the experiment it was noted that (1) cells, or cell groups, of the 2 species were intermingled apparently at random throughout the aggregates: at least there were no patches of red Microciona cells or of colorless Cliona cells, but the aggregates were of a uniform, intermediate color; (2) the aggregates had a somewhat porous structure; in the reticulum between pores, the cell-to-cell contacts seemed to be about as close as in either Cliona or Microciona in normal serum.

EFFECT OF CALCIUM ON REAGGREGATION

Galtsoff (1925) and de Laubenfels (1932) have reported that reaggregation is impeded in media outside a limited range of calcium concentrations. But Agrell (1951) was unable to maintain *Halichondria panicea* suspensions as isolated cells in citrate or oxalate solutions. In view of this inconsistency, and considering the apparent role of surface antigens, it seemed desirable to check some of the previous work on the action of calcium. Three types of experiments were carried out: in calcium-free media, in the presence of a calcium-complexing agent, and in high calcium solutions.

Effects of calcium-free media. Galtsoff (1925) reported that in NaCl or KCl solutions, the cells do not move and no coalescence occurs, and that even after 24 hours, the substratum is covered with single cells which die about a day later.

Following Galtsoff's methods a piece of Microciona weighing two grams was squeezed through bolting cloth into 20 ml. of 0.52 *M* NaCl (isotonic). The suspension was centrifuged at 62 G for five minutes and the supernatant drawn off with a pipette; fresh solution was added and the tube containing the cells vigorously shaken. The centrifuging and subsequent washing were repeated three times. Two ml. of this suspension were added to (1) eight ml. of 0.52 *M* NaCl, to (2) eight ml. of 0.52 *M* KCl, and to (3) eight ml. of normal sea water, in syracuse dishes. After 24 hours, the number of aggregates, per microscope field, was counted for five random fields.

Medium	1	2	3
Average no. of aggregates per field	0	0	4.2

At 24 hours, the NaCl and KCl cell suspensions were swirled and large macroscopic aggregates were formed. Identical results were obtained if the cells were suspended for 24 hours in an isotonic NaCl solution and then washed with subsequent centrifuging, three times, re-suspending each time by vigorous shaking. It is clear, therefore, that the cells fail to reaggregate not because they were dead or lacked adhesiveness after 24 hours, but because they had lost the ability to move.

Effects of Versene on reaggregation. It might be argued the NaCl or KCl solutions do not completely deprive the cells of calcium, and in order to check the above observations, it was desirous to find some means of effectively binding calcium specifically, so that it would no longer be available to the cell. Sodium oxalate or sodium citrate can combine with calcium to form an insoluble precipitate but this might disrupt the internal organization of cells and consequently bring about their death. It was necessary, therefore, to use a substance which is capable of forming a strong soluble complex with calcium and which is not toxic to living organisms. Versene (ethylenediamine tetra acetic acid) is such a compound; at pH 7.0 it sequesters calcium from solutions in a 1:1 molar ratio, by chelation.

Two grams of Microciona were squeezed through bolting cloth into 20 ml. of 0.05 *M* di-sodium versenate in 0.50 *M* NaCl, 0.01 *M* phosphate buffered at pH 7.00. The suspension was centrifuged at 62 G for five minutes and the supernatant removed with a pipette; fresh solution was added and the tube containing the cells vigorously shaken. The centrifuging with subsequent washing was repeated three times. It was calculated that all of the calcium and possibly some magnesium ions should at that point have been chelated with Versene. Two ml. of the suspension were then added to (1) eight ml. of the di-sodium versenate solution and to (2) eight ml. of normal sea water, in syracuse dishes. After 24 hours the dishes were examined under the microscope and the aggregates counted.

The same results as in NaCl and KCl were obtained.

Medium	1	2
Average no. of aggregates per field	0	4.5

At this time the bottom of the versenate dish was covered with single isolated cells which did not exhibit either ameboid movement or coalescence. When the dish was swirled large macroscopic aggregates were formed. Identical results were obtained with cells first suspended for 24 hours in di-sodium versenate and then centrifuged followed by washing three times, re-suspending each time by vigorous shaking. Since a normal aggregate count was obtained with cells transferred to sea water, it is readily apparent that the cells remained alive in the versenate solution.

The above experiments offer additional evidence to support the conclusion that calcium deficiency prevents reaggregation by the inhibition of ameboid movement and does not affect or alter the adhesiveness of the cells to each other.

Effects of calcium excess on reaggregation. Although no role of calcium in cell adhesion is indicated by the effects of calcium deficiency, one might be detected by the effects of calcium excess. Therefore, one gram of Microciona was dissociated into 40 ml. of normal sea water and two ml. of the suspension were added to eight ml. of normal sea water, and to seven ml. normal sea water plus one ml. of 0.35 *M* $CaCl_2$ in syracuse dishes.

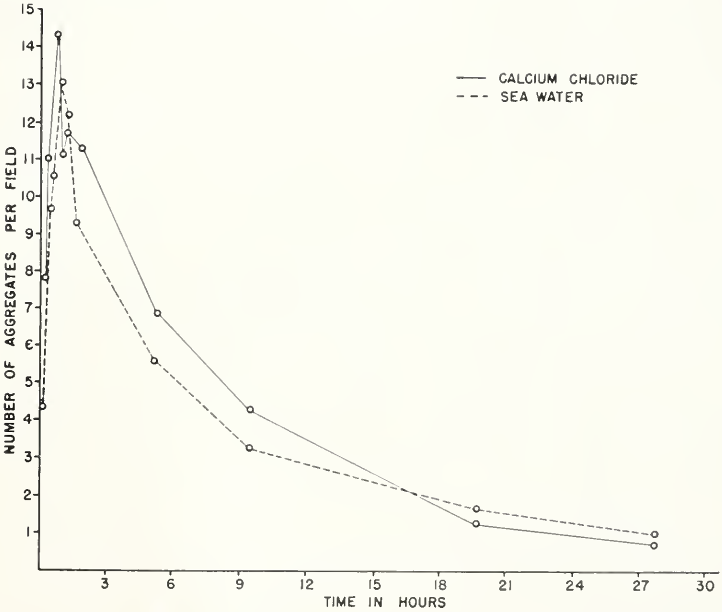


FIGURE 5. Reaggregation of Microciona in normal and high calcium media.

Aggregate counts were made at varying intervals; the reaggregation curves are shown in Figure 5. Differences are well within the limits of variation among preparations in sea water.

In experiments under identical conditions, Galtsoff (1925) found 7.1 times as many aggregates (meaning less reaggregation) in excess-calcium solution as in normal sea water at 24 hours. The difference in results is difficult to explain; it leaves considerable doubt whether reaggregation is significantly affected by increasing the calcium of the medium 4.1 times.

DISCUSSION

The present results show that reaggregation is inhibited by the presence of homologous antibodies. The usual expectation, when a specific antiserum is added to a suspension of the homologous cells, is that an agglutination of the cells will occur. The first point to be discussed, then, is why such agglutination by antibodies does not appear in the present experiments. There are at least three conditions under which agglutination may not occur even though specific reaction takes place between surface antigens and homologous antibodies:

(1) *Extreme antibody excess.* Under this condition the cells are, presumably, completely saturated with agglutinin before cell-to-cell contacts are made. The cells are incapable of reacting with each other since all their receptor sites are occupied. Such a hypothesis has been offered by McKerns and Denstedt (1950) to account for the invariable persistence of unagglutinated red blood cells in agglutinated samples even when potent isoagglutinating serum is used. Failure of agglutination in antibody excess is, of course, the expectation from the Marrack-Heidelberger-Pauling "mutual multivalence" hypothesis (see Boyd, 1947 for ref.). It does not seem likely, however, that the sera used in the present investigation are of such high titer that the vast majority of cells are saturated before cell collisions take place.

(2) *Univalent antibodies.* In this case it would be assumed that the antibodies formed by a rabbit in response to injections of sponge antigen are chiefly of the univalent type. These antibodies could combine with surface antigens but since each molecule of antibody possesses only a single group reactive with antigen no agglutination takes place. This type of antibody seems to be commonly formed in Rh antisera (Wiener, 1944) and other immune antisera (see refs. in Tyler, 1945). Such a hypothesis is adequate for explaining inhibition of sponge reaggregation by homologous antiserum but offers no explanation for the apparent agglutination of mixed suspensions of cells by the homologous antiserum.

(3) *Structure of cell surface.* Professor Albert Tyler (personal communication) has suggested a third condition under which agglutination may not occur. One may assume that the surface of the sponge cell is a folded, and probably also flexible, structure. Most of the surface antigens would then be so situated that the two or more valence groups of a single antibody molecule would tend to react with antigen on the same cell. Since the surface of a sponge cell is observed to change its shape, as evidenced by pseudopodium formation, it seems reasonable to assume that there is enough flexibility and movement so that the valence groups of the antibody molecule would be able to contact two or more of the receptor sites on the same cell surface. Very few, if any, valence groups would then be available for reaction with receptor sites on other cells. This, then, can account for absence of agglutination by homologous antiserum and the inhibition of reaggregation. It can also account for the clumping obtained with mixed cells (of the two species of sponge) in antiserum vs. mixed cells. Here it is assumed that heterovalent antibodies are formed. Considering (for simplicity) only two valences on the antibody molecule, one may be termed anti-M, the other anti-C. Such antibody molecules would react at one end with cells of one species of the mixed suspension (say M cells) leaving a valence group (anti-C) free to contact the cells of the other species.

The failure of ostensibly multivalent antibodies to cause agglutination has been noted in several immunological systems. Gleeson-White *et al.* (1950) and Coombs *et al.* (1951) have studied the reactions of ox red cells in rabbit or guinea-pig anti-ox sera. The red cells of most oxen fail to agglutinate in such sera. Coombs *et al.* suggest that the failure is due to a deep location of the antigens so that a single antibody molecule, even if multivalent, after combining with one cell cannot reach receptors of a second cell.

Coombs *et al.* have been able to accomplish the agglutination by certain devices which, in effect, can be considered to supply links between the antibody molecules attached to the different cells. For example, anti-rabbit globulin serum (produced in goats) will agglutinate the ox cells that have been treated with the rabbit anti-ox cells antiserum. This is particularly effective when the procedure is a serial addition of anti-globulin, then globulin, then anti-globulin, which is pictured as forming a longer chain, and provides strong support for the hypothesis of "deeply located" antigens.

This hypothesis does not, however, enable us to account for the agglutination of mixed cells by anti-mixed cells serum. It was therefore modified as described above in terms of interaction of a single antibody molecule with different parts of the same cell surface. Apart from the validity of the interpretation it appears clear from the work of Coombs *et al.* that there can be failure of agglutination, even in presence of multivalent antibody, with certain types of cells.

The effects of antisera on sponge reaggregation in relation to the Tyler-Weiss hypothesis will be considered in a stepwise discussion of the results.

1. *Anti-Microciona serum inhibits reaggregation of dissociated Microciona.* This conclusion can scarcely be doubted from the joint evidence of reaggregation curves up to three hours in standard and alcohol-killed suspensions, aggregate counts at 24 hours, microscopic appearance and size of aggregates at 2.75 and 24 hours, in normal serum and antiserum.

2. *The primary inhibiting effect of antiserum is on the process of adhesion.* The initial step in reaggregation, ameboid movement producing random contacts between cells, is normal in antiserum. But even when cells make contact in antiserum they fail to coalesce. There is no implication here of the mechanism by which the area of contact increases such as was suggested by Schmitt (1941).

3. *Some substance or class of substances "in" the cell surface is essential to the process of adhesion.* This conclusion is based on three considerations. (i) The zipper action, by which two impinging cells increase their area of contact, leads into the persisting contacts between cell surfaces within an aggregate. (ii) It seems probable that the same forces are responsible for the initial adhesive and the permanent contacts. The probability of this proposition is difficult to estimate for sponges because the boundary which can be seen, while two cells are joining, disappears within a few minutes. It is this change to which Galtsoff (1925) applied the term coalescence. Apparently the two hyaloplasms merge and the factors responsible for binding the two cells together then become simply part of the structure of the hyaloplasm. (iii) The *intrinsic* factors that hold cells together are commonly thought to be in the cell surface, *e.g.*, lipids. For many types of cells, extrinsic factors have been shown to be equally important, *e.g.*, calcium in salt linkage between lipids or proteins of adjacent cell surfaces, or "intercellular cement" which may involve a similar chemical bounding of an extrinsic substance to surface materials of two cells (Chambers, 1940). Other possible extrinsic factors are materials such as histones (Schmitt, 1941).

4. From steps 2 and 3, *the primary effect of antiserum is on some substance or substances in the cell surface.* Adhesions of two cells must involve interactions of their surface. It seems extremely unlikely that the antibodies affecting adhesion

would enter the cell producing some change inside which is later manifested in altered properties of the surface. A direct action of antibodies on the surface is more reasonable and can explain the results.

5. From the steps 1 and 4 it is reasonably probable that *anti-Microciona serum blocks the normal action of the materials in the cell surface that are essential for adhesion.*

6. *The only substances in anti-Microciona serum that are not also present in normal serum are antibodies to substances of Microciona that can act as antigens.* This has a three-fold basis. (i) Three anti-Microciona sera gave positive precipitin tests with Microciona extracts; two other anti-Microciona sera had the same effects on reaggregation as the three with precipitin tests. Therefore all five almost certainly contained antibodies. (ii) Three normal sera gave negative precipitin tests with Microciona extracts; two others, not precipitin tested, had no effect on reaggregation nor did the three tested. Therefore all five almost certainly had no antibodies to Microciona. (iii) Antisera do not normally differ qualitatively from normal sera, where comparable animals are used, except by presence of antibodies (Boyd, 1947).

7. From steps 5 and 6, *the normal role of certain surface antigens in adhesion is blocked by homologous antibodies present in antisera to Microciona cells.* The antigens will be represented by the symbol M, and the antibodies by anti*M*.

8. From comparable, less extensive tests, and by similar reasoning, *antigens A, in the surface of Cliona and essential in adhesion, are blocked in function by antiA in anti-Cliona serum.*

9. *M and A are specific antigens.* The main evidence here is that anti-Cliona serum does not block reaggregation of Microciona; the reasoning is analogous to steps 1–7. With Cliona in anti-Microciona serum there is some inhibition of reaggregation. One possible explanation for this is that Microciona possesses below the cell surface an antigen immunologically similar to a surface antigen of Cliona.

10. *M is inactive with respect to adhesion because it is combined with antiM.* This follows from step 7 and from the general knowledge that the only significant action of antibodies, under circumstances analogous to the present, is to combine with their homologous antigens (Boyd, 1947). The compound will be represented as M-anti*M*, without any implication as to the actual number of molecules of either M or anti*M* in the compound.

11. *M is not completely removed from a blocked cell.* This must be concluded from the fact that reaggregation can occur after washing an antiserum-treated suspension in sea water. It does not seem likely that such washing dissociates an antigen-antibody complex since such dissociation evidently involves more drastic treatment, such as high salinity (Heidelberger and Kabat, 1937) or high pH (Liu and Wu, 1938; Sternberger and Pressman, 1950). A more likely interpretation is that M extends below the surface; perhaps the entire hyaline layer is a gel of M and that M, at the surface, normally goes slowly into solution in sea water and is continuously replaced by M manufactured below. On this basis then, M-anti*M* would leave the surface slowly, or more rapidly by washing the cells, which would then be capable of reaggregating.

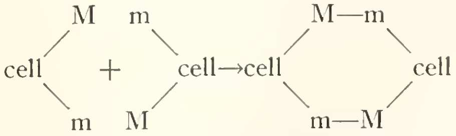
12. *The region of the M molecule to which antiM attaches is either* (a) *the site that is involved in adhesion, or* (b) *near enough to that site so that antiM overhangs or somehow interferes with the activity of the adhesion site.*

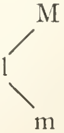
This is based on step 11 and 3 other considerations. (i) There seem to be only two possible ways of operation of M in normal adhesion: either through an extrinsic binder like calcium, *e.g.*, cell-M-Ca-M-cell; or by some type of direct bonding between M of one cell and M or some other substance m of another cell, *e.g.*, cell-M-m-cell, as envisaged by Tyler and Weiss. In this case a single cell can be assumed to have both M and m on the surface. (ii) The former seems unlikely considering particularly the failure of Versene to prevent reaggregation and the apparent absence in Microciona of anything able to act as an intercellular cement. De Laubenfels (1932) cites some evidence suggesting that, in dissociated *Iotrochota birotulata*, an intercellular slime is dispersed throughout the medium and brings cells or cell groups together, presumably by some sort of contraction. The writer has not seen, or had evidence of, any such material in Microciona. When a suspension is stirred the cells or cell groups move independently of each other; there are no signs of intercellular traction, nor any visible strands that could be identified as slime. (iii) Conclusion 12 follows from the remaining possibility: that the process of adhesion is essentially cell-M + m-cell → cell-M-m-cell.

13. *Alternatives a and b of step 12 cannot be readily distinguished.* The antibodies might well be directed against some constituent of the surface that is not directly concerned in reaggregation. Such bound antibodies might partly overlie the chemical sites involved in adhesion. This possibility cannot be distinguished on the basis of present evidence from the possibility that the antibodies combine directly with the chemical sites involved in adhesion.

14. It is likely that *adhesion depends on the presence, in the cell membranes, of two intra-molecular configurations with the reciprocal structural relationship of antigen and antibody.* It is possible that the reciprocals exist either within a single molecular species or in distinct substances. For the reactant reciprocal to M, there will be retained the symbol m introduced at the end of step 12 without implying that these sites are identical with the antigenic sites against which the antibodies are directed. Adhesion implies that M and m are each present in the cell surface in such position and arrangement that M and m of one cell can, under proper circumstances, combine with m and M of another cell. If this is so, within the surface of any one cell M and m must be so held in position that they cannot combine with each other.

The structure of a cell surface, with respect to adhesion, would then be crudely analogous to a balloon on which many snap-fastener parts have been attached, male and female separately but interspersed. And the *unit* reaction in adhesions would be



The M-m bonding would be that of antigen to antibody; the cell  bonding would be whatever holds the components of a cell surface in position.

Is this necessarily a correct picture? Obviously a yes-or-no answer can not be given. At best one might hope to calculate a probability that it is: one would have to estimate the probability of correctness at each step in reaching the final conclusion, and multiply those component probabilities together. Here too, it seems impossible to give a specific answer.

One conclusion of some immunological interest seems inescapable; that, when Microciona and Cliona cells are injected together into a rabbit, some antibody molecules are produced with one or more groups reactive to M or m *and* one or more groups reactive to A or a. These may be termed mixed or heterovalent antibodies. If such antibodies are formed—presumably along with homovalent anti*M*, anti*m*, anti*A*, and anti*a*—two opposing processes will together determine what happens when a mixture of Microciona and Cliona cells is put into anti-Microciona: Cliona serum:

1. blockage of M, m, A, and a, by the homovalent antibodies;
2. linkage of heterovalent antibodies to M or m of a Microciona cell *and* to A or a of a Cliona cell.

Thus of the total antibody combined with M or m, a certain proportion is heterovalent, with a second group that can react with the remaining uncombined A or a. This should lead to aggregation between cells of the two species as described in the fourth paragraph of the discussion.

Examination of standard texts and monographs does not give the non-immunologist any clear impression whether or not it is reasonable to account for the present results as has been done, in terms of heterovalent antibodies. Burnet and Fenner (1950) say (p. 36) "There is fairly conclusive evidence that an antibody cannot function as such against two different antigens," referring to work of Dean, Taylor and Adair (1935).

Roesel (1951), on the other hand, has obtained evidence indicating that heterovalent antibodies are produced in the rabbit in response to injections of a mixture of the bacteriophages T_2 and T_5. Race, Sanger, and Lawler (1948) believe that the majority of anti-C (anti-rh′) sera are really anti-C + C^w in specificity since either antigen, C^w or C, is capable of removing both antibodies. They feel that both components, anti-C and anti-C^w are on the same molecule.

There is therefore some evidence in favor of heterovalent antibodies and the assumption of such antibodies offers a satisfactory explanation of the experimental results obtained with mixed cells in mixed antiserum.

The author wishes to express his deep appreciation to Professor Donald R. Charles under whose guidance the work was performed. He is also indebted to Professor Albert Tyler for aid in editing the manuscript.

Summary

1. An investigation of the substances that bind adjacent cell surfaces together is reported here, using two species of sponge, *Microciona prolifera* and *Cliona celata*. Antisera were made, in rabbits, to cell suspensions of each species and to a mixture of cells of both species.

2. Reaggregation of dissociated cells was reversibly inhibited in the homologous antiserum. In normal serum containing cells of both species, the cells sort out to

form aggregates consisting entirely of either one species or of the other, *never* of both species. In an antiserum vs. both species, large aggregates were observed which consisted of cells of both species distributed at random throughout each aggregate.

3. Calcium-free and high calcium media had no effect on reaggregation.

4. The results are compatible with the Tyler-Weiss hypothesis that contiguous cell surfaces are normally held together by forces like those between antigens and homologous antibodies.

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THE ROLE OF SPECIFIC SURFACE ANTIGENS IN CELL ADHESION. PART II. STUDIES ON EMBRYONIC AMPHIBIAN CELLS[1]

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Holtfreter (1943) first demonstrated that embryonic amphibian cells could be disaggregated by exposure to his standard solution (Holtfreter, 1931) at high pH. Townes (unpubl.) has utilized this technique to investigate the problem of cell affinities. The following account is based on these previous investigations.

If an amphibian gastrula or neurula is exposed to Holtfreter's solution at a pH greater than 9.6, the pigmented surface coat becomes soft and is partially dissolved. "Within a few minutes cracks appear in it; new disruptions follow, until the whole surface is broken up into small dark islets, surrounded by much larger white interfaces. A closer examination reveals that each islet is the centre of a group of cells, attached to it in a radial orientation" (Holtfreter, 1943; p. 292).

Under the action of the alkali, the surface coat, which is disrupted at the cell boundaries, retracts in the form of a black cap to the pole of each cell. This cap progressively decreases in size as pigment streams to the interior of the cell.

Within 20 minutes, the entire gastrula or neurula is a heap of dissociated spherical cells which can be separated from one another with a glass needle. If the solution is now removed and replaced with 2–3 changes of Holtfreter's solution at pH 7.8, the entire process is reversed.

The cells begin to adhere to one another immediately after the removal of alkali, and within 0.5–1 hour have formed a mass of irregular form. At 3–8 hours, partly depending on species used, the aggregate has rounded up into a smooth solid ball of cells in intimate contact. Ectoderm cells, at first randomly distributed within the aggregate, have migrated to the exterior and formed sheet-like patches among the endoderm, at the end of 24–72 hours. If sufficient ectoderm cells are supplied they will almost completely cover the aggregate. Mesoderm cells are chiefly found between the ectoderm and endoderm, with a few scattered among the endoderm cells (Townes, unpubl.). The surface coat is reconstituted and the entire mass is usually covered with pigment. During this period intercellular spaces appear due to the secretion of an interstitial fluid. It appears to be identical with blastocoel fluid, having the same property of reducing the mutual adhesiveness of the cells (Holtfreter, 1944). Smaller aggregates lack the interior intercellular spaces altogether.

If an aggregate is allowed to develop further, differentiation proceeds and may include development of neural folds, eyes, ears, beating heart, etc. (Townes, unpubl.).

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Holtfreter (1939a, 1939b) has shown that small pieces of ventral ectoderm and endoderm, brought into contact, at first cling together but later tend to separate into discrete masses. The self-isolation begins after 2 days in *Rana pipiens* and 3–4 days in *Ambystoma punctatum*, and is complete after 4 and 6–9 days, respectively. However, if mesoderm is combined with ectoderm and endoderm, self-isolation does not occur; the mesoderm behaves as a binding agent.

These tissue affinities are apparently not species-specific at early stages of development, and may be seen in practically any xenoplastic combination. For example Townes (unpubl.) has found that the ectoderm of *Rana pipiens* gastrula with the endoderm of *Ambystoma punctatum* or *Triturus torosus* will exhibit self-isolation. If, however, mesoderm is included, an aggregate is formed which holds together.

With these observations and experiments as a basis it seemed desirable to test the effects of antisera on the reaggregation, extending thus the previously reported (Spiegel, 1954) experiments on dissociated sponge cells.

EFFECTS OF ANTISERA ON REAGGREGATION AND SEGREGATION

Materials and Methods

Experimental induction of breeding activity. Fertilized eggs of *Rana pipiens* were obtained through ovulation by hypophysis injection, and artificial insemination, as described by Rugh (1948). *Triton alpestris* eggs were very kindly furnished by Dr. J. Holtfreter.

Preparation of protein extract. Ten grams of the plant enzyme papain were extracted with 500 ml. of 10% Holtfreter's solution (3.5 grams NaCl, 0.05 gram KCl, 0.1 gram $CaCl_2$, 0.2 gram $NaHCO_3$, per liter distilled water) for six hours at 4° C. (Spiegel, 1951). The solution was filtered, 300 mg. cysteine.HCl added, and the pH adjusted to 6.6 with 0.1 *N* NaOH and 0.1 *N* HCl. Stage 12 (Shumway, 1940) gastrulas, 3410 in number, were placed in an eight inch finger bowl, the bottom of which was lined with ¼ inch of 2% agar, and covered with 500 ml. of the papain solution.

After three hours, the two outer layers of jelly had dissolved; the eggs were washed twice with 10% Holtfreter's solution, and 500 ml. of 2.5% sodium thioglycolate (in 10% Holtfreter's solution, pH 8.1) added. In ten minutes the vitelline membrane and third jelly layer were dissolved. The eggs were washed with three changes of 0.65% NaCl (0.01 *M* phosphate buffered at pH 7.3), without being allowed to come in contact with an air-water interface.

Protein was extracted in the medium used by Gregg and Ballentine (1946) (0.65% NaCl in 0.01 *M* phosphate buffer at pH 7.3), 65 ml. of solution being used for the 3410 eggs. The suspension was homogenized by hand for five minutes at 3–4° C. in a Ten Broeck glass homogenizer. A drop of the homogenate was examined under the microscope and no intact cells were noted.

The homogenate was kept at 4° C. for sixteen hours, with frequent shakings, then centrifuged at 600 G and the cloudy supernatant collected. Nutrient and blood agar plates streaked with the extract showed slight bacterial contamination. Therefore, 0.02 mg. Streptomycin ($CaCl_2$ complex) and 0.02 mg. Penicillin G potassium were added to each 50 ml. supernate to prevent bacterial growth and the extract was frozen in liquid air and stored at − 20° C. until use. The nitrogen content of the extract, determined by micro-Kjeldahl, was 1.4 mg. N/ml. extract.

Twenty-four of 25 control eggs from the same clutch, and with jelly and vitelline membrane removed, developed normally to stage 22.

Preparation of antiserum. One male New Zealand Giant rabbit received eight subcutaneous injections, 0.5 ml. extract per injection, every fourth day (Cooper, 1948). A second uninjected male served as control. Seven days after the last injection, the animals were bled by cardiac puncture and the sera recovered. Both sera were heated to 56° C. for 0.5 hours to inactivate complement. Serum obtained from the animal injected with gastrula extract will henceforth be called anti-gastrula serum.

Precipitation tests were set up by layering 0.1 ml. of undiluted extract and of 11 two-fold dilutions (1:2 to 1:2048 in saline) over 0.1 ml. of antiserum, or over saline or normal serum as controls, in 10 × 75 mm. serological tubes. All tubes were incubated at 37° C. for one hour and then examined for presence of a precipitate ring at the interface. The antiserum produced rings of antigen-antibody complex at all antigen dilutions down through 1:1024. Controls were negative.

Tests of antiserum effects on reaggregation. For experiments dealing with ectoderm and endoderm, the membranes were removed with fine forceps from stage 10 or 11 *Rana pipiens* embryos, or the dorsal lip stage of *Triton alpestris,* and the desired tissues were isolated with glass needles in Holtfreter's solution. If mesoderm was to be used in addition to ectoderm and endoderm, the tissues were isolated either from stage 12 *Rana pipiens* embryos or from the dorsal lip stage of *Triton alpestris.*

The isolates were transferred by pipette to a syracuse dish containing 10 ml. sterile Holtfreter's solution, with 2% agar as substratum, and 1% KOH was added dropwise until disaggregation was complete. Occasionally, a clear viscous, somewhat elastic material or slime could be seen in the disaggregate; such preparations were rejected with one exception. The cells were washed 4–6 times with sterile Holtfreter's solution. Finally 10 ml. of normal serum or anti-gastrula serum, diluted with distilled water to give a final salt concentration of 0.38%, were added. By use of glass needles the cells were pushed into a heap with ectoderm, mesoderm, and endoderm cells randomly distributed.

RESULTS

Twenty different combinations of tissues, isolated cells, antiserum, normal serum, and Holtfreter's solution were examined.

Effect of Rana pipiens anti-gastrula serum on disaggregated whole Rana pipiens embryos. Stage 9, 10 and 12 embryos were disaggregated and placed in anti-gastrula serum. After 1.5 hours the reaggregation, if any, was slight. At 7–14 hours the cells had adhered and sorted out to some degree, but remained spherical with conspicuous intercellular spaces. Endoderm cells in particular were loosely packed. Some slime was noted between the cells. In the stage 12 disaggregate, it appeared as though ectoderm cells would adhere to ectoderm, but not to endoderm, when pushed into contact. Endoderm cells, on the other hand, would adhere to endoderm, but not to ectoderm.

In contrast, the cells of disaggregates of stage 9 and 12 embryos, in *normal* serum, adhered to each other at the end of four hours, and the aggregate began to round up. At eight hours, the aggregates were well rounded with interspersed patches of ectoderm and endoderm cells forming a smooth surface. At 15 hours the coat was being reconstituted. No masses of slime were noted.

Effect of Rana pipiens anti-gastrula serum on disaggregated ectoderm, endoderm, and mesoderm cells of Rana pipiens. After one hour in anti-gastrula serum, about 100 disaggregated mixed ectoderm, mesoderm, and endoderm cells, from a stage 12 embryo, had partially adhered. The ectoderm and endoderm cells appeared to have sorted out, but were loosely packed. No change was noted after this period.

Effect of antiserum on disaggregated ectoderm and endoderm cells of Rana pipiens. After 3.5 hours in antiserum, approximately 100 disaggregated ectoderm and endoderm cells, from a stage 11 embryo, adhered to each other, but the aggregate failed to round up. At four hours, the cells remained spherical and the aggregate surface was not smooth. Ectoderm and endoderm cells appeared randomly dispersed. No slime was noted. At 65 hours the ectoderm and endoderm cells still had not sorted out, and the surface was unchanged.

Ectoderm and endoderm cells of stage 11, after one hour in *normal* serum, had adhered and the surface was becoming smooth. By four hours, the aggregate had rounded up and ectoderm and endoderm cells had sorted out. The cells at the surface were flattened and a smooth exterior was noted at 17 hours. By 65 hours, the ectoderm and endoderm formed two separate aggregates connected by a short stalk of cells.

Effect of Rana pipiens anti-gastrula serum on disaggregated endoderm and ectoderm cells of Triton alpestris. Approximately 130 ectoderm and endoderm cells of the dorsal lip stage of *Triton alpestris* were disaggregated and placed in *Rana pipiens* anti-gastrula serum. After one hour, the cells had adhered to each other, with surface cells flattened and in intimate contact. At 17.5 hours the aggregate had become spherical with smooth surface. No slime was noted. At six

TABLE 1

Summary of experiments showing effects of frog anti-gastrula serum on reaggregation of dissociated frog and salamander cells

	Mixtures of disaggregated ectoderm and endoderm cells				Mixtures of disaggregated ectoderm, mesoderm, and endoderm cells			
	Normal serum		Antiserum		Normal serum		Antiserum	
Condition	2R*	1T**	1R	1T	3R	1T	5R	1T
Time (hours)	17	17	28	18	8	16	14	16
Aggregates rounded:	+	+	−	+	+	+	−	+
Exposed cell surfaces are flattened:	+	+	−	+	+	+	−	+
Random intermixture of ectoderm and endoderm cells:	−		+		−		±	
Time (hours)	65	144	65	144	36	144	36	144
Ectoderm and endoderm in separate aggregates:	+	+	−	+	−	−	−	−

* Number of experiments with cells from *Rana pipiens* embryos (R).

** Number of experiments with cells from *Triton alpestris* embryos (T).

days the ectoderm and endoderm cells had sorted out to form two completely separate aggregates.

Ectoderm and endoderm cells in *normal* serum sorted out after six days to form two aggregates connected by a short stalk.

Effect of Rana pipiens anti-gastrula serum on disaggregated mesoderm, ectoderm, and endoderm cells of Triton alpestris. About 130 ectoderm, mesoderm, and endoderm cells of *Triton alpestris*, dorsal lip stage, in *Rana pipiens* anti-gastrula serum formed smooth surfaced aggregates after one hour and the aggregate became spherical by 16 hours. No slime was noted. Cells in *normal* serum behaved in identical fashion.

The results, summarized in Table I, indicate strongly that, in embryonic amphibian cells as in adult sponge cells, specific surface antigens are an essential part of the mechanism by which adjacent cell surfaces are bound together during reaggregation.

DISCUSSION

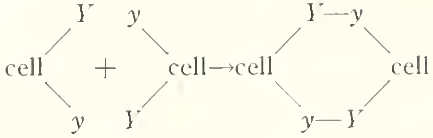
The results with embryonic amphibian cells are difficult to explain except in terms of the Tyler-Weiss hypothesis that contiguous cell surfaces are normally held together by forces similar to those between antigens and homologous antibodies (Tyler, 1940, 1942, 1946, 1947; Weiss, 1941, 1947, 1950).

As in the case of cell adhesion in sponges described by Spiegel (1954) the results show that reaggregation of embryonic cells is inhibited, in contrast to the usual phenomenon of agglutination, by the presence of homologous antibodies. Spiegel (1954) has presented three conditions under which such inhibition may occur: (1) extreme antibody excess; (2) univalent antibodies; (3) structure of cell surface. Only the third condition will be discussed here (see Spiegel, 1954, for a discussion of conditions 1 and 2).

It has been suggested by Professor Albert Tyler that perhaps the cell surface of embryonic amphibian cells (and also of sponge cells) is a flexible, folded structure with the specific surface antigens so situated that the two or more valence groups of a *single* antibody molecule would tend to react with antigen on the *same* cell. Only a few, if any, valence groups would be free to react with receptor sites of other cells and thus would account for the absence of agglutination. It is, of course, also assumed that the reaction between specific surface antigens either blocks the adhesion sites or in some manner (*i.e.*, steric interference) interferes with them. The interpretation of the failure of the antibodies to agglutinate the sponge cells or the embryonic amphibian cells is a modification of the hypothesis presented by Coombs *et al.* (1951) to account for failure of homologous multivalent antibodies to agglutinate the red cells of most oxen. In their view a deep location of the antigens is assumed so that a multivalent antibody molecule after combining with one cell cannot reach receptors of a second cell.

That these surface antigens are specific has been demonstrated by the ability of cells of *Triton alpestris* embryos to reaggregate normally in *Rana pipiens* anti-gastrula serum.

The Tyler-Weiss hypothesis, as interpreted by Spiegel (1954), implies that adhesion depends on the presence, in the cell membranes, of two intra-molecular configurations with the reciprocal structural relationship of antigen and antibody. These exist either within a single molecular species or in distinct substances. If the two reciprocal configurations are designated by Y and y, then the *unit* reaction in cell adhesion would be



As a logical consequence of this hypothesis, the segregation of ectoderm and endoderm cells from each other, in Holtfreter's solution or normal serum, into two separate aggregates, would indicate that different surface antigens are present on cells of each of the two tissues. Recent evidence obtained by Clayton (1953) with *Triton alpestris* embryos, seems to indicate that ectoderm and mesoderm, at least, contain different antigens. However, endoderm antigens were not investigated and the location of these antigens in the cell was not determined.

The fact that mixtures of ectoderm, endoderm, *and* mesoderm cells, in Holtfreter's solution or normal serum, round up to form a single aggregate consisting of three concentric layers of cells, with the mesoderm layer situated between ectoderm and endoderm (as in normal development), suggests an interesting possibility with regards to the surface antigens of mesoderm. Perhaps mesoderm, in addition to possessing specific surface antigens which are important for the adhesion of mesoderm cells to each other, also possesses sites which are complementary to ectoderm surface antigens *and* to endoderm surface antigens. Thus, in effect, mesoderm would act as a heterovalent antibody (a multivalent antibody molecule, with *different reactive sites,* which are capable of combining with at least two different antigenic groups) to ectoderm cells and to endoderm cells, and would be capable of binding the two cell types together. This would be analogous to the hypothesis proposed by Spiegel (1954) for the failure of segregation of mixtures of the cells of two species of sponge in antiserum produced in rabbits by simultaneous injection of cells of *both* species. Here it was proposed that *the rabbit* formed heterovalent antibody to the surface antigens of cells of both species and thus such antibody molecules were able to agglutinate both cell-species.

Each of the amphibian results is what would be expected from the sponge experiments performed by Spiegel (1954). Amphibian and sponge experiments, together, can be explained on the basis that the ability of cells to recognize each other depends upon the presence, in their surfaces, of specific antigens which also bring about the normal adhesion of cells, by reciprocal reactions similar to those between antigens and their homologous antibodies.

The author would like to express his gratitude to Prof. Donald R. Charles under whose guidance the work was carried out. He is also indebted to Prof. Albert Tyler for his aid in the editing of the manuscript.

SUMMARY

An investigation of cell adhesion in embryonic amphibian cells is reported here. Antiserum was made in rabbits vs. a cell extract of stage 12 *Rana pipiens* embryos. The reaggregation of dissociated cells of Rana ectoderm and endoderm was in-

hibited by the antiserum. The antiserum had no effect on the reaggregation and segregation of dissociated cells of *Triton alpestris* embryos. The role of antigen-antibody-like reactions in cell adhesion is discussed.

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STUDIES ON THE CARDIAC STOMACH OF THE STARFISH, ASTERIAS FORBESI[1]

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The peculiar feeding methods of starfishes have attracted attention since at least the time of Aristotle. Problems involved in explaining the various phenomena of the feeding process have been investigated repeatedly, yet in many cases the solutions advanced for the problems are less than satisfactory. For example, "the old starfish-clam question," of the methods by which the starfish gains access to the interior of a bivalve, still awaits a convincing answer (see Reese, 1942). Also deserving of closer scrutiny is the mechanism by which food is brought into the digestive tract of the starfish. As Tiedemann (1816) points out, many of the ancients held the erroneous belief that the starfish ingested the soft parts of snails and bivalves by exerting suction. Müller (1788–1806?)[2] and Cuvier (1805) are cited by Tiedemann as the first to call attention to the characteristic eversion of the cardiac stomach of *Asterias*, a process which in its superficial aspects has been described many times in the years since Tiedemann's monograph appeared.

The general mechanism bringing about eversion of the cardiac stomach is now rather well understood, owing in part to Cuénot's account (1887); however, it is apparent that many features of the retraction of the stomach after feeding remain to be explained. The tough strands connecting the cardiac stomach with the ossicles in the floor of each ray have been variously referred to as mesenteries, as tendons, as strands of connective-tissue infiltrated by muscle fibers, and as retractor muscles, revealing a fundamental uncertainty as to their actual function. In addition, the relationships between these strands and the wall of the stomach have apparently never received an adequate description.

The present report is an attempt to interpret the results of a series of dissections and histological studies on the structure of the cardiac stomach of *Asterias forbesi*, particularly as these results are related to observations and preliminary physiological experiments on the mode of operation of the retractor system. Attention is also directed toward the relationship which exists between the distribution of retractor fibers and certain other specific structural features of the wall of the stomach. No attempt will be made to explore the subject of the digestive and absorptive functions of the cardiac stomach, although these functions are imperfectly understood and require further study.

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[1] The studies on which this report is based received support from the Sarah Manning Sage and Dr. Solon P. Sackett Research Funds of the Department of Zoology, Cornell University.

[2] Tiedemann's reference is to Vol. IV, p. 13, of Müller's *Zoologia Danica*; he does not specify a date. I have assigned the dates of the only 4-volume edition which appeared prior to Tiedemann's *Anatomie* but have been unable to check with the original.

Material and Methods

The animals used in these studies were vigorous specimens, ranging from 5 to 8 inches in over-all diameter. They were maintained in running sea water at Woods Hole, or in 15-gallon marine aquaria at Ithaca. The starfishes were fed periodically with pieces of the soft parts of the quahaug, *Venus mercenaria*, or with chunks of frozen whiting fillets, previously thawed. No difficulty was encountered in inducing the animals to feed, once they had become acclimated to conditions in the aquaria. It should be noted, however, that the starfishes generally ignored the living quahaugs kept with them in the tanks; only once during a period of several months was a quahaug successfully attacked and opened by one of my starfish.

Preparation and treatment of specimens for preliminary physiological experiments will be described below, as necessary, in connection with the observations. For histological work, the central part of the alimentary tract was exposed by removing from the living animal, relaxed by exposure to sea water containing added $MgSO_4$, the aboral walls of the disk and of the proximal parts of the arms. The pyloric stomach and rectal caeca were discarded, and the portion of the cardiac stomach pertaining to a single arm was carefully excised by radial incisions through the wall of the stomach, and by cutting across the peristome. The extended attachments of the retractor strands to the ambulacral ossicles in the floor of the arm were severed, and the portion of the stomach thus freed was transferred to a petri dish of sea water with added $MgSO_4$, for further relaxation. In this manner, the cardiac stomach of a single specimen could be removed in 5 approximately equal parts. Larger pieces could not be easily manipulated during the following operation, which consisted of carefully spreading and pinning out the segments of the stomach in small, wax-bottomed dishes containing a little sea water. These spread segments were studied from either the inner or the outer surface. Suspensions of india ink in sea water, placed dropwise on the inner surface, facilitated observation of the currents maintained by the ciliated (or flagellated) cells of the lining epithelium. Study of the outer surface permitted determination of the precise courses of the retractor fibers upon and within the wall of the stomach.

Following such gross observations, the sea water was decanted from the spread preparations, and the tissue, still pinned in place, was flooded with one or another histological fixative. After partial fixation had occurred, the pins were removed, and small, carefully oriented parts of the stomach were excised and transferred to fresh fixative. After imbedding in paraffin or in gelatin, these smaller parts were sectioned in planes parallel with, or perpendicular to, the plane of the mouth.

Serial sections 5 μ in thickness, of material fixed in Zenker-formol, were stained in a variety of ways for over-all orientation and to permit the differentiation of muscular, collagenous, and elastic fibers in the retractor mechanism. Most useful in this connection were Mallory's phosphotungstic acid hematoxylin, Krichesky's modification of the Mallory triple connective-tissue stain, van Gieson's picro-acid fuchsin collagen method, and the Taenzer-Unna acid-orcein method. Feulgen preparations were also employed, as well as the periodic acid-Schiff routine for the demonstration of glycogen and other polysaccharide compounds. Dilute toluidine blue was used to localize metachromatic elements. To reveal the distribution of lipids, material fixed in Baker's formol-calcium and imbedded in gelatin was sectioned on the freezing microtome, colored with sudan black, and counterstained with carmalum.

One additional type of preparation was found useful to reinforce anatomical interpretations from gross dissections. From a starfish 6 inches in diameter, one arm was removed, together with a portion of the disk containing the part of the stomach attached to that arm. The aboral body wall was removed, and the pyloric stomach, pyloric caeca, and gonads discarded, along with the distal two-thirds of the arm. The remaining piece consisted of the floor of the proximal portion of the arm, the floor of a pie-shaped segment of the disk, and that part of the cardiac stomach attached to this piece by its complete retractor system and by the peristome. This preparation, designed to show the relationship between the normal, retracted cardiac stomach *in situ* and its retractor harness, was fixed in Zenker-formol. The tissue was subsequently decalcified for 48 hours in a 10% aqueous solution of disodium versenate,[3] dehydrated, and imbedded in paraffin. Serial cross-sections were prepared at 10 and 15 μ, and the sections were stained as in the case of the spread segments of the stomach described above.

OBSERVATIONS

A. The retractor system

As revealed by aboral dissection, or by a sagittal section of an arm, what might be termed the *extrinsic* part of the retractor mechanism of the cardiac stomach consists of 5 pairs of triangular, mesentery-like sheets, one pair in each arm, which connect the wall of the stomach with the ambulacral ossicles in the floor of the arm. One side of each triangle is formed by a strand originating distally in the floor of the arm, usually in the neighborhood of the 30th pair of ossicles. The other side is represented by a similar, but shorter, strand which originates from the lateral aspect of the first ambulacral ossicle. As shown in Figure 1, these strands appear as the thickened, free, upper borders of a long, low, triangular, mesentery-like sheet of tissue, which binds the strands to the series of ambulacral ossicles throughout their length; the extended attachments of this sheet to the ossicles form the base of the triangle.

Microscopically, the strands are found to consist chiefly of fibrous connective tissue, both collagenous and elastic, mingled with rather scanty muscle fibers. The sheet below the strands also contains collagenous fibers which run obliquely upward from the sheaths of the ossicles and interdigitate with the fibers of the strands. The strands and the fibrous sheet are enclosed by a fold of mesothelium, continuous with the peritoneal lining of the body wall.

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[3] An organic chelating agent, the di-sodium salt of ethylene diamine tetra-acetic acid. It has been successfully used in the demineralization of tooth and bone in vertebrates (see, for example, Birge and Imhoff, 1952, and Freiman, 1954) but to my knowledge has not previously been applied to calcareous tissues in invertebrates. Its advantage over the classical mineral-acid decalcifying procedure lies in the fact that the versenes apparently do not destroy residual enzymatic activity and other characteristics of tissues, of interest in histochemical studies. The samples of disodium versenate employed in these investigations were generously supplied by the manufacturer, Bersworth Chemical Co., Framingham, Mass.

The apex of the triangle is the point at which the four fibrous strands in each arm are bound together by a transverse, band-like connection (Figs. 1 and 2). This general region will be referred to as the *nodule*. It is a tough, fibrous structure attached to the outer surface of the stomach by many radiating bundles of fibers. The nodule projects from the surface of the stomach, and when the stomach is fully retracted usually lies above the 10th to 12th ambulacral ossicles.

Each of the 5 nodules may be considered as forming a point of transition between the *extrinsic* retractor mechanism in the arms, just described, and the *intrinsic* part of the system, which lies upon and within the wall of the stomach. Passing through the nodular region, the extrinsic strands coalesce and then give rise to a complicated system of branching fibers radiating over the wall of the stomach. From each nodule arise two major bundles which proceed in opposite directions, in a plane generally parallelling that of the oral opening. Each of these major bundles gives off secondary branches, some of which are distributed over the roof of the cardiac stomach, but most of which proceed downward toward the mouth. These downward-coursing fibers branch in an apparently specific fashion, tapering gradually throughout their length. They enter the wall of the stomach, running under its peritoneal covering, and contribute fibers to its muscular and connective-tissue layers. Their ultimate branches may be traced, in sections, as far as the distinct line, above the mouth, which appears to mark the junction between stomach and

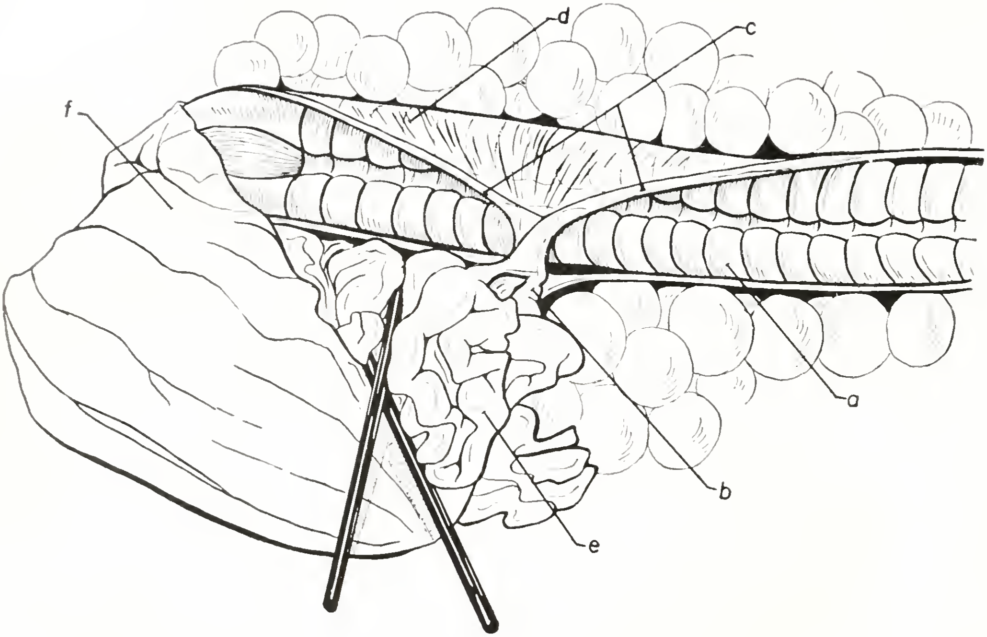


FIGURE 1. Dissection of an arm of *A. forbesi* to show extrinsic retractor mechanism. At *a*, ambulacral ossicles; *b*, region of the nodule; *c*, proximal and distal extrinsic strands; *d*, mesentery-like sheet; *e*, outer surface, and *f*, inner surface of a segment of the cardiac stomach, pinned aside.

esophagus. The branching patterns of the terminal fibers coincide in a consistent manner with corresponding series of branching channels, to be described presently, which are characteristic of the lower portion of the wall of the stomach.

Histologically, the nodules and the intrinsic retractor strands may be described as variable mixtures of collagenous and muscular fibers, with an elastic fiber component throughout but concentrated mainly in the nodule and its major branches. The nodule appears as a band of complexly wound collagenous bundles, interspersed with smaller muscle fibers and bound together by branching strands of elastic tissue. In cross section, the large and moderate-sized bundles arising from the nodule appear to consist of a central, collagenous band, around which are arranged in a radial pattern 6 or 8 smaller, more compact bundles of collagenous fibers (Fig. 4). Aggregations of muscle fibers occupy the spaces between the collagenous strands, and the entire ensemble is enclosed by a mesothelial covering. In the smaller branches, farther removed from the nodule, the proportion of muscle fibers increases. These small branches eventually run under the peritoneal layer and often appear in sections as local thickenings of the outer coats of the stomach wall. They can be recognized, however, by the characteristic arrangement and direction of their fibers. Such a small branch appears as a central cord of collagenous tissue surrounded by larger or smaller groups of muscle fibers (Fig. 4, arrow).

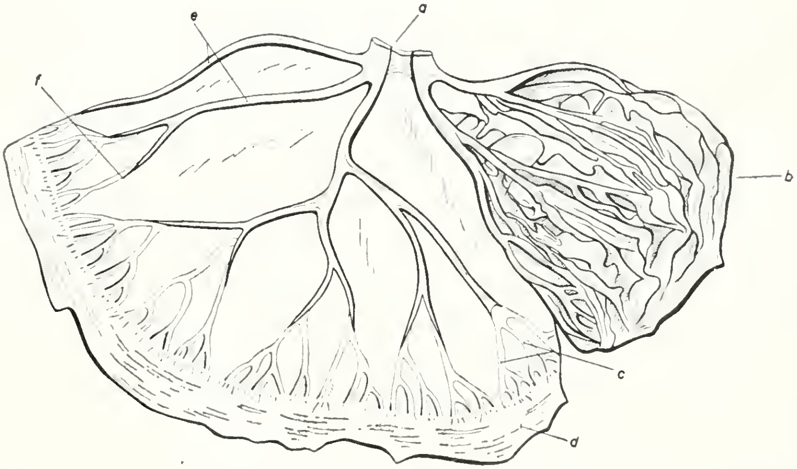


FIGURE 2. Excised and partially spread segment of cardiac stomach, as viewed from its outer surface. At *a*, region near the nodule; *b*, unspread portion of the stomach; *c*, branches of a channel system seen through wall of stomach; *d*, esophagus; *e*, intrinsic retractor fibers; *f*, point of entry of fibers into wall of stomach. As shown in the unspread portion at the right of the figure, the esophagus normally gathers the lower end of the stomach like a draw-string. It must be cut and stretched considerably to allow spreading of the stomach on a flat surface, as at the left.

B. The structure of the wall of the stomach

In its general histological composition, the wall of the cardiac stomach in the starfish is substantially as described for various genera, including *Asterias*, by

Hamann (1885), Cuénot (1887), Ludwig (1899), Schneider (1902), Chadwick (1923), and Hayashi (1935). There is considerable variation in details between various genera. In *Asterias,* the innermost layer is a pseudostratified, columnar epithelium composed of tall, slender cells interspersed with mucous gland cells and secretory cells containing coarse granules; the distribution of these glandular cells varies in different parts of the stomach. Between the elongated bases of the epithelial cells run the fibers of nerve cells constituting the "nerve layer" (Chadwick, 1923) or "neurofibrillar plexus" (Smith, 1937), varying in thickness in various regions. The extended basal processes of the epithelial cells rest upon, and often appear to intertwine with, the fibers of the connective-tissue layer lying just outside the neurofibrillar plexus. Beneath this collagenous and elastic coat (Fig. 4) are two layers of muscle fibers; the fibers of the inner layer take a generally circular course, while those of the outer layer are longitudinal. The longitudinal muscle layer is covered externally by the visceral peritoneum, which, as elsewhere in the body, consists of a single layer of approximately cuboidal, flagellated cells. The connective-tissue layer appears to serve as the basement membrane of the epithelium and throughout its distribution sends short, branching fibers into the nerve layer and into the muscular layers. These fibers appear to be elastic strands which aid in maintaining the spatial relationships between the connective-tissue layer, the epithelium, and the muscular coats.

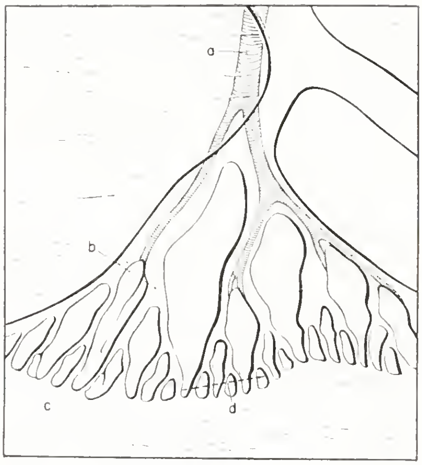


FIGURE 3. Portion of a channel system as viewed on the inner surface of the spread stomach. At *a,* intrinsic retractor bundle seen through the wall of the stomach; *b,* channels; *c,* esophagus; *d,* approximate plane of section shown in Figure 5. The eminences or ridges between the terminal branches of the channel patterns are covered with the closely packed specialized epithelial cells.

The most conspicuous structural feature of the cardiac stomach, viewed either in spread preparations (Figs. 2, 3) or in cross-sections (Fig. 5), is the series of branching systems of channels, or gutters, beginning at the line which marks the oral end of the cardiac stomach. The broad, widely-dispersed lower ends of these systems of grooves open on this line of demarcation; the gutters pass upward, converging in a specific pattern which appears to follow generally the convergent paths of the retractor fibers described above. In the portion of the stomach pertaining to each arm there are usually 10 sets of these gutter systems. The smaller end-branches of the patterns, within the mouth, are deeper and narrower than the larger branches, higher in the stomach, formed by coalescence of the smaller channels. In its finer details, the distribution of the gutter patterns does not exactly follow the branching patterns of the retractor fibers, but there is a high degree of correspondence between the two systems.

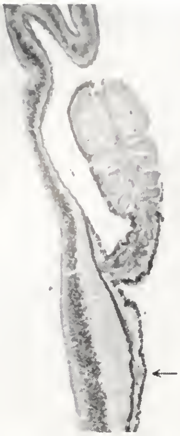
Figure 3 shows a series of these gutter patterns as they appear on the inner surface of a spread segment of the cardiac stomach. The distribution of the related retractor fibers in the outer layers of the stomach wall is also indicated. Figure 5 shows a cross-section of the wall of the stomach through a portion of a single gutter system. It should be noted that the grooves follow a specific pattern, and that the alternating channels and ridges involve not the epithelium alone, but the entire thickness of the wall of the stomach.

As shown in Figure 6, the epithelium lining the bottom and sides of a single groove is composed of tall, slender, closely packed cells, each containing a small, ovoid nucleus. This kind of cell may be termed "typical" in appearance; that is, cells like these predominate in the epithelium of the cardiac stomach as a whole. Each of these typical cells bears a single, long flagellum with a single basal granule, and the distal ends of the cells are covered by a well developed brush border.

As Figure 7 shows, an entirely different kind of cell, which we may term a "specialized" cell, occurs on the ridges between adjacent grooves. Such a specialized cell is characterized by a thin, elongate nucleus occupying one-third to one-half of its length. The specialized cell bears more than one flagellum, the number varying between two and six. Each flagellum springs from a separate basal granule, and coarse, deeply staining fibrils from these granules coalesce to form a single strand which appears to join the nuclear membrane, or to proceed beside the nucleus toward the base of the cell. The elongated nuclei of the specialized cells are much denser and less granular than the ovoid nuclei of the typical cells; the Feulgen stain reveals the long nuclei as dense, homogeneous masses of DNA.

In addition to the specialized cells, the ridges between the channels bear many large mucous gland cells. The ovoid nuclei of these gland cells lie near the base of the epithelium, and the mucous goblets stream upward toward the free border, often compressed and distorted by the crowding of adjacent cells. At the lower end of each ridge there occurs a rather gradual transition to the type of epithelium characteristic of the esophagus; here the specialized cells are not so closely packed but are interspersed with very numerous mucous gland cells and cells filled with coarse, refringent granules.

A particularly sharp line of transition between the typical cells and the specialized cells occurs partway up the side of each ridge; this may be noted in Figure 5 and is shown more clearly in Figure 8. Below the line of transition, that is, nearer the bottom of the groove, there appears a band of typical cells which present interesting distal differentiations. These cells are distinguished by the fact that their distal ends contain masses or strands of small granules, lying about and below the basal granules of the flagella. The small granules appear to stream out of the cells of their origin and are scattered about in and on the brush border of neighboring cells. The general appearance of such a region of cells with distal differentiations



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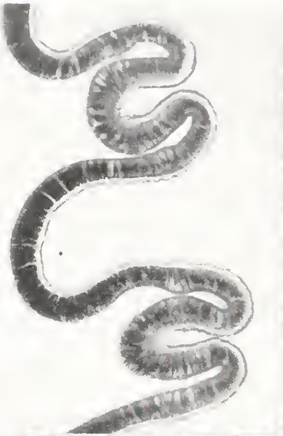

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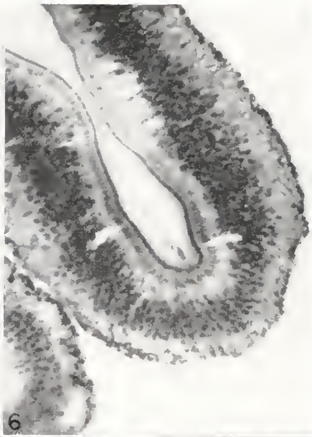

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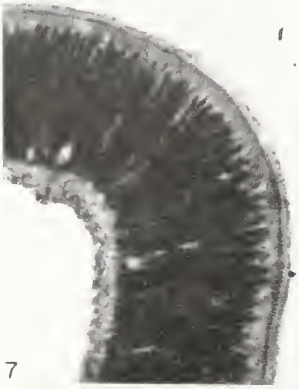

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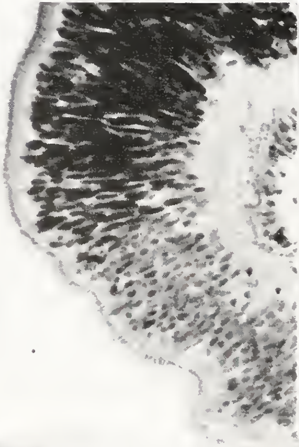

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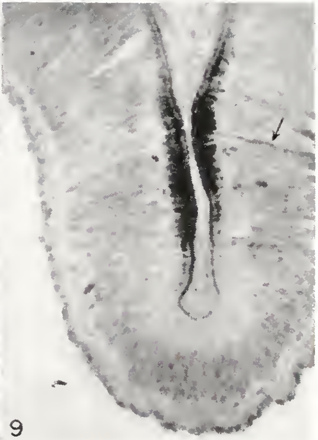

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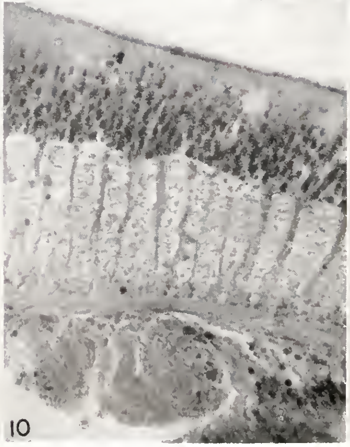

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PLATE I

is shown in Figure 9. The small granules stain metachromatically with toluidine blue; they react positively with the periodic acid-Schiff routine, and this property is not destroyed by exposure to diastatic digestion. In addition, they stain deeply with acidulated orcein. In all respects except this last, they behave like the secretion of the mucous gland cells, but in their form and distribution they are very different from the mucous secretions. Small aggregations of similarly metachromatic and Schiff-positive secretion products occur in many of the typical cells throughout the lining of the cardiac stomach, but in the restricted, band-like areas of the walls of the grooves they are much more concentrated.

Figure 9 also shows the appearance of a type of gland cell not previously described. It occurs always in company with "typical" cells; its nucleus is located basally, and the shape of the cell is always like that of a flask with an extremely long and attenuated neck, extending to the free border of the epithelium. The finely-granular, dense cytoplasm of such cells stains deeply with phosphotungstic acid hematoxylin and with acidulated orcein, unlike ordinary mucous goblets. These cells may represent early stages in the secretory cycle of mucous gland cells.

The epithelium of the region below the ends of the groove patterns, which I have interpreted as corresponding to the esophagus, has a finely corrugated appearance. The corrugations (Fig. 3) involve only the epithelial and nervous layers, not the entire thickness of the wall. They are arranged in a circular direction, and this portion of the wall seems capable of considerable distension. The epithelium of the esophagus consists of specialized cells with long nuclei, like those covering the ridges at the lower end of the stomach. In this upper portion of the esophagus the specialized cells are accompanied by very numerous granular secretory cells and many mucous gland cells. There is a gradual transition to lower, more nearly

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Plate I

With the exception of Figure 5, all figures in this plate are of material fixed in Zenker-formol, sectioned at 5 μ, and stained as indicated. Magnifications given are approximately those of the figures as they appear here, after enlargement and reduction.

Figure 4. Longitudinal section through upper region of stomach, showing a fairly large retractor fiber bundle in cross-section. Lumen to left. Mesothelial covering has been lost from outer surface of bundle. Note thickness of nerve layer; at arrow, small retractor fiber under peritoneum. Acidulated orcein, hematoxylin counterstain. 88 ×.

Figure 5. Frozen section, approximately 10 μ, through channel pattern at lower end of stomach (see Fig. 3, *d*). Lumen to left. Note contrast in epithelium in channels and on ridges. Sudan black, carmalum, after formol-calcium fixation. 52 ×.

Figure 6. Epithelium at base of channel. Note small, ovoid nuclei; brush border; basal granules and distal fibrils in cells. Light areas are mucous gland cells. Phosphotungstic acid hematoxylin. 278 ×.

Figure 7. Epithelium on ridge between channels; lumen to right. Note dense, elongate nuclei. Small clear areas are mucous gland cells. Phosphotungstic acid hematoxylin. 378 ×.

Figure 8. Transition zone between typical cells and specialized cells on the side of a ridge. Note multiple basal granules and distal fibrils in specialized cells; comma-shaped nuclei in these cells are not intact but have been cut at an angle. Phosphotungstic acid hematoxylin. 387 ×.

Figure 9. Typical cells with distal differentiations, in sides of a small channel. Note that similar areas in Figure 6 have not stained with hematoxylin. At arrow, flask-shaped gland cell. Acidulated orcein, no counterstain. 278 ×.

Figure 10. Epithelium at region of attachment of a retractor strand; *cf.* Figure 4. Note arcades formed by bundles of basal processes from epithelial cells; fibers and nuclei in nerve layer; absence of specialized cells from epithelium in this region. Lumen above. Phosphotungstic acid hematoxylin. 443 ×.

typical epithelial cells in the lower regions of the esophagus, and the secretory cells decrease in numbers.

The areas of the wall of the cardiac stomach lying between and above the orally directed groove patterns are covered by an epithelium which consists entirely of typical cells, interspersed with mucous gland cells. My studies indicate that the specialized cells with elongate nuclei are found nowhere in the wall of the cardiac stomach proper, except on the ridges separating the channels near the esophagus. The coarsely granular secretory cells appear never to occur in the cardiac stomach itself along with typical cells; they are found in the esophagus only, and only in company with specialized cells.

In regions adjacent to the attachment or insertion of retractor fibers, higher in the stomach, the nerve layer is often considerably thickened. In such areas (Figs. 1, 10), the epithelial cells send down bundles of elongate, fibrous basal processes which insert on the connective-tissue layer. These bundles form arcades through which course the fibers of nerve cells. Preliminary results of histochemical studies show that in well-fed animals droplets of lipid and deposits of glycogen are localized in the tapering portions of these epithelial cells, just above their fibrous processes.

C. Physiological studies

On the surface of the esophagus, on the ridges, and on the inner surface of the cardiac stomach generally, the currents produced by the vibratile organelles of the epithelial cells sweep in the direction from mouth to anus. Within the branching channels, however, the only currents I have been able to demonstrate are those which might be termed *centripetal*; that is, particles coming into a channel are whisked out of it again, toward the center of the digestive cavity.

Following the injection of 25 ml. of a 1:10,000 sea water solution of acetylcholine chloride into the perivisceral coelom of a large starfish, it is possible, by exerting pressure on the arms, to bring about the eversion of the cardiac stomach to any desired degree. Observation of the extruded vesicles of the cardiac stomach indicates that the major part of the "ballooning" of the stomach wall occurs in the region of the so-called esophagus. The region of the stomach marked by the channel systems appears to slide more or less straight downward from the mouth and may be protruded to a considerable degree. The channels at the lower ends of the systems may extend laterally a short distance over the lower, medial ends of the vesicles, but for the most part the channel systems lie on the inner faces of the vesicles, surrounding the narrow passageway leading upward into the stomach. It is likely that contributions to the stretched walls of the vesicles are made by the thin portions of the stomach wall lying between the separate sets of gutter systems.

The demonstration that acetylcholine facilitated the artificial eversion of the stomach, which cannot be caused at will in a normal, untreated animal, made it desirable to determine in a preliminary manner the effects of acetylcholine and of adrenalin on the various parts of the stomach involved in eversion and retraction. Accordingly, several starfishes were prepared by removal of the aboral wall of the disk and excision of the pyloric stomach and the roof of the cardiac stomach. In this way the inner surfaces of the cardiac stomach and of the esophagus were exposed, the closed mouth appearing at the center, below the esophageal area. The folds of the retracted stomach, together with the retractor harness, remained intact. A solution of acetylcholine in sea water (1:10,000), dripped slowly on the region surrounding the mouth internally, caused a slow, steady, considerable enlargement of the oral opening. If the acetylcholine was flushed away with fresh sea water, and a 1:10,000 sea water solution of adrenalin chloride was dripped on the area around the open mouth, the oral opening immediately and rapidly closed. This alternate opening and closing of the mouth under the influence of the two drugs could be repeated many times, the solutions being washed away each time with fresh sea water. Little, if any, effect of either drug could be noted when solutions were applied to the folded walls of the retracted stomach, or to the retractor strands. Minute changes in the length of the extrinsic strands extending into the arms would be difficult to detect under these conditions; various small movements of the underlying body wall do occur, and these are reflected in small, apparently passive changes in the tension of the strands.

Discussion

Knowledge and understanding of the retractor mechanism of the cardiac stomach have had a slow growth and a varied history. Linck (1733; Appendix IV, p. 100, first commentary on Kade's para. IX) makes an indirect reference to 5 connecting ligaments in relation to the stomach. Cuvier, writing about the year 1800, in his description of the nervous system of the starfish, writes as follows (Ross' translation, 1802, Vol. II, p. 369): "Round the oesophagus we observe a girth of a soft whitish substance, which produces ten filaments, two to each of the branches . . . the two filaments belonging to each branch having arrived at the base of the osseous and articulated stalk . . . unite to form a short cord, which extends directly from the one to the other; . . . At the place where they are united, each produces a fasciculus of filaments, which are distributed to the stomach. . . . The appearance of all these filaments is rather tendinous than nervous, and that circumstance has hitherto chiefly prevented us from forming a decided opinion of their nature." [4] From this description, one is struck by the accuracy of Cuvier's observations; he is clearly describing the intrinsic and extrinsic retractor mechanisms of the cardiac stomach. And, obviously, he was not convinced that these filaments represented the nervous system, although he discusses them in connection with the nervous system and does not suggest for them any alternative function.

With less caution, Spix (1809), a disciple of Cuvier, gives a less accurate description of the retractor system of the stomach in *Asterias rubens*, stating that the nodules (Spix's own term: p. 440) on the wall of the stomach are ganglia, and that the various fibers and strands radiating from them are other parts of the nervous system.

Tiedemann (1816) corrects Spix's (and Cuvier's) error [5] and describes the counterpart of the fibrous system in *Astropecten aurantiacus,* where it differs in many respects from that of *Asterias.* (The differences are in part presumably related to differences in feeding habits: whereas *Asterias* carries on much of its digestive activity with the vesicles of the cardiac stomach everted, *Astropecten* apparently everts the stomach only to engulf food which is then "swallowed" by retraction of the vesicles; see, for example, Cuénot, 1891, p. 416; Ludwig, 1899, p. 733.) Tiedemann, although he knew well the eversible nature of the cardiac stomach, describes the fibrous strands as "tendinous" (*sehnenartige*) and does not impute to them any retractor functions. He says merely (p. 43): "All of these strands connect the outer surface of the stomach with the inner surface of the skin, and hold the stomach spread and suspended in its place." [6]

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[4] Cuvier's original statement (T. II, p. 360) is as follows: *Autour l'oesophage s'observe une ceinture de substance molle et blanchâtre, d'ou partent dix filets: deux pour chacune des branches. . . . Les deux filets qui appartiennent à chaque branche étant arrivés à la base de la tige osseuse et articulee . . . se réunissent par un cordon court qui se rend directement de l'un à l'autre. . . . A l'endroit où ils se réunissent, part de chacun d'eux un faisceau de filets qui se distribuent sur l'estomac. . . . L'aspect de tous ces filets est plutôt tendineux que nerveux, et c'est sur-tout cela qui nous empêche de nous décider encore.*

[5] It is somewhat puzzling to note that Tiedemann, who was an enthusiastic follower of Cuvier and familiar with his work, does not mention Cuvier's suggestion of the nervous nature of these fibers, although in an extended footnote he practically ridicules Spix for his similar interpretation.

Since the descriptions furnished by Cuvier and Spix, the pertinent literature reveals a surprising lack of interest on the part of investigators in what I have termed the intrinsic retractor mechanism. Ludwig (1899, p. 587) speaks of paired *Magenmesenterien,* arising from several roots on the outer surface of the stomach; here, however, as in most cases, the author deals almost exclusively with the nature and disposition of the extrinsic strands. With regard to these extrinsic parts, an interesting shift of opinion has occurred: their function as muscular retractor strands has been emphasized, while their fibrous nature, and the possibility that they may serve simply as ligamentous attachments, have been lost sight of. Hoffmann (1872, p. 6) writes of *Asterias rubens*: "The 10 flat bands which arise from the under surface of the stomach, run in pairs into the arms, and serve for the attachment of the stomach, consist of connective-tissue bundles infiltrated by muscle strands." [7] Cuénot (1887, p. 41) attributes the withdrawal of the stomach after feeding to the contraction of muscles in the mesenteric strands (". . . *pour le réintégrer, les muscles des brides mésentériques se contractent, et l'attirent à l'intérieur*"). Cuénot's 1887 opinion is essentially restated in his last work on this subject (1948). Ludwig describes the extrinsic retractors as mesenteries and states clearly that they are composed of connective-tissue fibers, with only a slight representation of muscular tissue. Chadwick, in his monograph on *Asterias* (1923), shows by a figure (Plate V, Fig. 43) that he had observed the true appearance of the triangular extrinsic sheets, with the nodules at their apices; however, he considers the strands as retractor *muscles* and gives no indication of their composition, or of their possible function as simple attachments for the stomach. He writes (p. 20): "The stomach is furnished with five pairs of retractor muscles. . . . These muscles come into action when the cardiac sac is retracted after eversion. . . ." Modern British and American textbook authors unanimously follow Chadwick and speak of the strands as retractor muscles, which by their contraction withdraw the stomach after feeding.

As the earlier authors were aware, and as indicated by the present study, the histological structure and the anatomical relationships of the extrinsic strands and sheets do not appear to justify this interpretation. Each of the nodules on the wall of the stomach is bound to the ambulacral ossicles of its arm by 4 tough, collagenous strands, which are further secured by the mesentery-like sheets. Comparison of a series of preserved specimens with stomachs in different stages of eversion or retraction reveals that the nodules do move in relation to the ossicles, but only very slightly; in the most strongly everted specimen the nodules lay over the 4th and 5th ossicles. These facts, and the very scanty musculature of the extrinsic parts of the system, make it clear that the principal function of the extrinsic strands and sheets is to provide a firm but elastic and slightly movable anchorage for the nodules, and through the nodules, for the intrinsic retractor fibers and the wall of the stomach itself. Against these 5 anchored points the branching and widely distributed strands of the intrinsic retractor system may passively stretch and pull, during eversion, as the mouth opens and the increased intracoelomic pressure inflates and everts the thin-walled pockets of the stomach.

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[6] Tiedemann's statement (p. 43): *Alle diese Faden verbinden die äussere Fläche des Magens mit der inneren Fläche der Haut, und erhalten den Magen ausgebreitet und aufgehängt in seiner Lage.*

[7] Hoffmann writes (p. 6): *Die 10 platter Bänder welche von der unteren Fläche des Magens entspringen, paarig in die Armen verlaufen und zur Befestigung des Magens dienen, bestehen aus mit Muskelfasern durchsetzten Bindegewebsbündeln.*

The fact that the nodules do move during eversion, and that the extrinsic strands do contain muscle fibers, suggests that these parts possess a slight contractility useful in returning the nodules to their normal positions at retraction. The more significant aspects of retraction, however, involving the deflation and withdrawal of the vesicles and the folding of the walls of the stomach into their positions within the disk, undoubtedly depend upon the contractility and elasticity of the intrinsic retractor mechanism. In the constituent strands of this system, as in the wall of the stomach itself, muscular fibers are well developed. The proximal ends of these fibers are ultimately attached to the relatively fixed nodules; their distal ends insert on the walls of the cardiac stomach in widely distributed patterns near the esophagus. Contraction of these fibers would therefore "pucker" the walls of the everted vesicles, reducing their volume and forcing the return of their fluid contents into the perivisceral coelomic cavities of the arms. The specificity and constancy of the patterns of distribution of the intrinsic retractor fibers make it apparent that the folding of the wall of the stomach is not a random process brought about by traction from the remote extrinsic parts, but occurs in an orderly, specific fashion, controlled by the intrinsic musculature.

The mouth is opened by relaxation of the fibers of the relatively thick circular muscle layer in this area, and contraction of the longitudinal muscle fibers, which in this region (peristome, *Mundhaut*) take a radial course. Cuvier (1837, T. V., p. 377) recognizes the reciprocal functions of the circular and longitudinal muscular layers in the control of the mouth, remarking that the opening and closing of the mouth resembles that of a pupil. The antagonistic effects of acetylcholine and adrenalin in controlling these processes may be assumed to be operative in the normal feeding activities. Previous investigators have demonstrated that muscular tissues of the alimentary tract in *Asterias* contain appreciable quantities of acetylcholine (Bacq, 1947) and exhibit marked cholinesterase activity (Augustinsson, 1946). The results of the present preliminary studies indicate that these chemical mediators operate in relation to specific processes involved in the normal functions of the alimentary system. It would be helpful to know whether the antagonistic effects of acetylcholine and adrenalin on the muscles of the oral region reflect reciprocal innervation of the circular and longitudinal muscle layers of the peristome from separate components of the nervous system. The details of the innervation of these muscular layers in *Asterias* are apparently unknown. Hamann (1885) describes very generally the origin of the continuous peristomial and visceral nerve plexus layer from the nerve ring bordering the peristome. The relationships of the visceral nerve layer to the other components of the "ectoneural system" are outlined and discussed by Ludwig (1899, pp. 547 *ff.*). Smith (1937) describes superficially the probable sensory relations of the gut wall, but the presence of specific sensory and motor pathways in the nerve layer of the alimentary tract has not been demonstrated. It is to be hoped that the elegant researches of Smith (1937, 1950) on the sensory-neuro-muscular aspects of the somatic parts of the starfish may soon be extended to elucidate the functional details of the visceral system.

Whatever the related facts of innervation and neurohumoral activation may be, and granting readily the preliminary and tentative nature of the physiological conclusions drawn from the present studies, the following resumé of events in eversion and retraction of the cardiac stomach seems justified on the basis of observations to date:

Eversion: (a) opening of mouth (cholinergic fiber activity?); relaxation of muscles in wall of stomach and in intrinsic retractor strands;
    (b) contraction of muscles in body wall, increasing intracoelomic pressure against relaxed walls of the stomach, which are forced out through mouth as fluid-filled vesicles.
Retraction: (a) relaxation of muscles in body wall, decreasing intracoelomic pressure;
    (b) contraction of muscles in wall of stomach and in retractor system, forcing coelomic fluid back into cavities of arms, deflating vesicles, and folding walls of stomach into normal internal positions; closing of mouth (adrenergic fiber activity?).

If one assumes a degree of elasticity in the body wall, relaxation of the body wall musculature would permit an active increase in the volume of the perivisceral coelom and thus by elastic recoil exert a certain amount of suction on the vesicles of the everted stomach. Whether or not this actually occurs, it could theoretically operate as an additional factor in the retraction of the stomach.

The significance of the specific and conspicuous patterns of channels on the wall of the cardiac stomach remains to be explained. It is surprising that they have apparently escaped description by previous observers, who have contented themselves by remarking generally on the folded nature of the walls of the retracted stomach. The oversight may be accounted for by the fact that the patterns are unsuspected when one examines the retracted stomach. Under normal circumstances, the investigator rarely sees a strongly everted stomach; the "humped" position of the body during feeding effectively conceals the details of the everted vesicles. They may be properly examined only when the cardiac stomach is removed from the animal and spread. At first glance, one would expect that such convergent grooves, leading upward from the esophagus into the stomach, would be of value in providing protected channels for upward-flowing currents. However, as experiments with india-ink suspensions indicate, the channels are the only places on the surface of the epithelium where currents do *not* flow upward.

The specialized cells clothing the ridges between these channels may be noteworthy in this connection. Such cells have never been described in the classical histological studies of the cardiac stomach. Smith (1937), in a report dealing primarily with the nervous system, noted similar cells in the cardiac stomach of

Marthasterias glacialis. He does not clearly describe their distribution, stating merely that they are to be found only in those parts of the stomach where there is developed an extensive neurofibrillar plexus. Admitting the lack of direct evidence, Smith nevertheless suggests that these cells with "cigar-shaped nuclei" may represent sensory receptor cells. "Their presence would, indeed, not be unsuspected in an evaginable part of the gut which is capable of being wrapped round the prey during the process of ingestion" (p. 146). In the stomach itself, as the present study shows, these specialized cells are wholly restricted to the regions of the gutter systems; they are found otherwise only in the epithelium of the esophagus, which appears to contribute very significantly to the evaginated vesicles. These cells show no evidence of secretory activity, and, aside from their peculiar nuclei, their only other distinctions from typical cells (*i.e.,* multiple flagella, with well developed intracellular fibrillar connections) might well be adaptations to sensory receptor functions. It may be suggested that the alternating grooves and ridges, which are fundamentally of the nature of folds in the stomach wall, may have developed in conjunction with the distribution patterns of the terminal fibers of the intrinsic retractor system, and in response to their folding actions. The specialized cells may simply form patches of sensory epithelium between the grooves. One might emphasize also the fact that although the regions bearing the grooves and their supposed sensory epithelial patches are not themselves strongly everted at feeding, they do form an almost continuous lining for the narrow passageway leading upward into the stomach, through which all ingested material must pass.

The distal differentiations of the otherwise typical cells in the side walls of the channels remain without explanation. The granules which they produce are apparently some type of secretion showing similarities to mucus, but differing in the details of their formation and appearance. The cells are always found in such positions that their secretions will be carried outward, away from the surface of the epithelium, by the centripetal currents produced in the channels. It is to be hoped that further studies of these cells, and of the nature of their secretion product, will clarify their functions.

Summary and Conclusions

Studies on the structure and functions of the cardiac stomach and its retractor mechanism reveal that the outer surface of the stomach is provided with a complex, specifically distributed system of tapering, branching fibers of muscle and connective tissue, inserting ultimately on the muscular and connective-tissue coats of the stomach wall. The origins of these fibers are from 5 band-like nodules, the points of attachment to the stomach of the paired retractor strands in the arms. These strands, the *extrinsic* parts of the retractor system, form the upper borders of triangular, mesentery-like sheets which bind them to the ambulacral ossicles throughout their length. The extrinsic parts, as reported by many early investigators, consist chiefly of collagenous fibers and contain very scanty musculature; they appear to move but little in eversion or retraction. It is suggested that they serve merely to anchor the 5 nodular regions of the stomach, against which the branching *intrinsic* retractor fibers of the wall of the stomach may stretch and pull at eversion. It is further suggested that the more significant aspects of retraction involve not the apparently very slightly contractile extrinsic parts of the system, but the widely distributed, muscular branches of the intrinsic mechanism. This interpretation is not in accordance with that of modern authors, who refer to the extrinsic strands as retractor *muscles* and disregard the intrinsic parts of the system completely. A brief summary of the development of this narrow emphasis is presented.

The terminal branches of the intrinsic fibers form patterns coinciding with a series of branching systems of channels on the inner surface of the stomach. These gutters open at the line of junction between stomach and esophagus and run upward, coalescing to form larger and broader channels higher in the stomach. The bottoms of these channels are lined by typical, non-specialized, tall epithelial cells, each with a small, ovoid nucleus and bearing a single flagellum. The ridges between adjacent grooves are covered by specialized cells, each with a long, spindle-shaped nucleus and bearing from two to six flagella. It is not unlikely that, as Smith has suggested, these cells represent sensory receptor elements. They are localized in a part of the stomach which, not itself strongly everted into the vesicles, forms the lining of the narrow gullet through which all ingested food must pass. Similar cells are found in the lining of the esophagus, which appears to form the major share of the vesicles of the everted stomach. The specialized cells are not found in the stomach above the lower parts of the channel systems.

Although the currents maintained over the lining of the stomach generally sweep from mouth to anus, within the gutter patterns the flagella whisk particles not upward, but outward into the lumen. Certain secretory cells localized on the walls of the gutters may contribute their secretions to these centripetal currents; their function is unknown.

Acetylcholine and adrenalin act antagonistically in controlling the opening and closing of the mouth. Acetylcholine opens the mouth by causing contraction of the longitudinal (radial) musculature of the peristome and esophagus; adrenalin reverses this action by bringing the circular muscles into contraction. It is tentatively suggested that these effects may reflect reciprocal innervation of these muscular layers from different components of the circumoral nerve ring. No action of chemical mediators has been demonstrated on the elements of the retractor systems, extrinsic or intrinsic.

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THE RHYTHMIC ACTIVITY OF THE QUAHOG, VENUS MERCENARIA, AND ITS MODIFICATION BY LIGHT[1, 2]

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The correlation of activities of animals in their natural environments with specific times of day, tide, and phases of the moon is an often-observed phenomenon. Also, it is a well-established fact that some of these activities, especially those correlated with the day-night cycle, persist even after the organism has been placed under conditions in which all factors known to stimulate the organism are kept constant (see reviews: Welsh, 1938; Park, 1940; Kleitman, 1949; and Webb, 1950). The cycles of activity which continue unaltered under constant conditions have been spoken of as endogenous or persistent rhythms as opposed to exogenous ones which continue only while the particular activating stimuli are being received by the organism. It has been suggested very strongly that the so-called endogenous rhythms are dependent on highly stable, inherent, physiological mechanisms or "internal clocks." However, the possibility that the animals that display persistent cycles of activity are actually reacting to some cyclically occurring physical forces of the external environment, in a manner not yet determined, has also been postulated by investigators.

Early in the present century, persisting or endogenous tidal cycles of activity were reported for certain marine animals. These papers have been reviewed by Brown, Fingerman, Sandeen and Webb (1953) who have demonstrated the existence of a persisting tidal rhythm superimposed upon the diurnal rhythm of color change in the fiddler crab, *Uca pugnax*. Moreover, these investigators have shown that in these crabs a semi-lunar or 14.8-day cycle of color change is the result of the precise relationship between the diurnal and tidal rhythms. More recent work illustrates that the rates of O_2-consumption of these crabs also reflect the presence of these rhythms (Brown, Bennett and Webb, 1954). Here it was found that the rate of O_2-consumption was generally greatest, other factors being equal, one to two hours before the times of low tides in the area from which the animals were collected. Again, it was clearly evident that the systematic moving of the tidal influence relative to the diurnal one produced characteristic patterns of O_2-consumption which recurred about every 15 days, or once every semi-lunar period. There was

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also a suggestion in the results of these studies that another cycle of O_2-consumption, a lunar or 29-day one, was present for this species.

The O_2-consumption rates of certain of the marine molluscs have also been correlated with environmental factors. *Patella, Mytilus,* and *Haliotis* were reported to have persisting rhythms of O_2-consumption under laboratory conditions (Gompel, 1937). Although the hourly data of these studies varied considerably, the highest rates of O_2-consumption were generally apparent about the times of high tides and lower rates were recorded at the times of low tides. More spectacular rhythms of O_2-consumption are described by Sandeen, Stephens and Brown (1954) for the marine gastropods, *Littorina littorea* and *Urosalpinx cinereus.*

Other molluscs exhibit tidal rhythms of motor activity when maintained under constant conditions. Bohn (1904) reported that *Littorina rudis* became active at 15-day intervals in the laboratory. In its natural habitat this snail is covered by water only during the semi-lunar high, high tides which normally occur about every 15 days. A persistent rhythm of locomotory activity related to the tides is displayed by *Nassa obsoleta* (Stephens, Sandeen and Webb, 1953). Also, the filtering rate of species of *Mytilus,* using colloidal graphite, varies rhythmically in phase with the tidal cycles in the area from which the mussels were collected (Rao, 1954).

Some of the characteristics of these persistent rhythms have been elucidated by various experimental procedures of which light and temperature modifications have been employed most often. The effects of changes in light on the diurnal rhythm of color change for fiddler crabs are discussed by Brown and Webb (1949), Webb (1950), Brown and Hines (1952), and Brown, Fingerman and Hines (1954). Brown and Stephens (1951) have reported modifications of this cycle which were induced by photoperiod. The only paper to date which describes the experimental modification of a tidal rhythm by changes in illumination is that of Brown, Fingerman, Sandeen and Webb (1953). These investigators found that when the diurnal color change rhythm for *Uca pugnax* is shifted by an appropriate light stimulus, the tidal rhythm is shifted correspondingly. Temperature studies have demonstrated that the frequencies of both the diurnal and tidal rhythms of color change for *Uca* are temperature-independent over fairly great ranges, at least 6° C. to 26° C. for the diurnal cycle (Brown and Webb, 1948), and at least 13° C. to 30° C. for the tidal cycle (Brown, Webb, Bennett and Sandeen, 1954). The tidal cycle of pumping for *Mytilus* is independent of temperature in the range 9° C. to 20° C. (Rao, 1954).

The remarkable stability of the so-called endogenous rhythms has supported the hypothesis that the possession of these cycles is of adaptive significance for these animals. Thus, they are able to anticipate the time of day or tide, or phases of the moon. Organisms whose feeding activity and/or breeding periods seem to be correlated with specific times in these cosmic cycles would appear to be likely subjects for the study of biological rhythms.

Korringa (1947) was able to show that the time of spawning of oysters and several other lamellibranchs exhibits a lunar periodicity. The results of studies on oysters (Korringa, 1952) and quahogs (Loosanoff, 1939) in their natural habitats, however, have been interpreted as indicating that these animals do not display any persisting activity correlated with the time of day or tide.

It was the purpose of the present study, therefore, to determine whether or not the common quahog, *Venus mercenaria,* an animal of the intertidal zone and shallow water, exhibits activity cycles under constant laboratory conditions. The activity rhythms that were observed have been related to the day-night cycle, to the phases of the tidal cycles, and hence to lunar periodicity. Also, an attempt was made to modify the character, or phase relationships to time of day, of the cycles of activity by light changes.

Material and Methods

The activities of three groups of quahogs were studied in Evanston, Illinois, during the winter and spring of 1953. They were obtained from a local fish market, and had been collected originally in the Chesapeake Bay region of Virginia. A fourth group was studied during June, 1953; these were clams which had been collected in Little Harbor, Woods Hole, Massachusetts. In December, 1953, the activities of two more groups of quahogs were studied concurrently in an attempt to ascertain the effects of light changes on the persistent cycles of activity. These animals again were obtained from a local market in Evanston, and had been collected in Virginia.

All the clams used for the activity studies were adults. There were eight to ten animals in each group; additional clams, which were obtained at the same time as those whose activity was being recorded, were kept under the same conditions as those that were being studied. In the event that a clam died, one of the additional animals was substituted, and the activity records of the original animal were discarded. Over the course of the observations, the mortality rate was very low. The great majority of the quahogs were in good condition even after having been in the laboratory for as long as four weeks.

The quahogs were placed in finger bowls containing sea-water which was changed at irregular intervals so that any observed rhythms could not be attributed to a regular time of water change. During the course of the study of the first four groups, the illumination at the surface of the water, as measured with a Weston photometer, was constant at approximately 2 ft. c. The source of illumination was fluorescent lights. The temperature of the water varied irregularly between 20° C. and 22° C.

Steel wires were bent to fit snugly over one of the valves of each animal from umbo to ventral edge. To these wires were tied threads which, in turn, were connected to sensitive heart levers or to the levers of the automatic recording spring scales described by Brown (1954). These levers recorded the opening and closing movements of the clams continuously on slow kymographs for periods of two to three weeks.

The daily record of each clam was analyzed by determining the times of opening and closing and the duration of the period open or active. The results obtained from the eight to ten animals of a group were averaged to obtain values for the total amount of time open per day. The time open was plotted against the days.

The following procedure was followed in the study of the effects of changes of illumination on the rhythms. One group of animals, the control group, was maintained under constant illumination of 2 ft. c. The experimental group was kept in a photographic dark room. For five consecutive nights (8 P.M. to 8 A.M.) this group was exposed to fluorescent lights providing an intensity of 100 ft. c. During the day (8 A.M. to 8 P.M.) they were kept in darkness. From 8 P.M. of the last day of darkness until the end of the experimental period, they were maintained at an illumination of 2 ft. c. During the three-week period, the temperatures of the two rooms in which the clams were kept were recorded continuously on Taylor thermographs, and were seen to fluctuate between 20° C. and 22° C. The records for these animals were analyzed as were those for the clams of the first four groups. However, the average values for three consecutive days were averaged and this average used as the value for the second of the three days, *e.g.,* the figures for December 4, 5, and 6 were averaged and the resulting value used as the time open for December 5. Such procedure was used to obtain the relationship illustrated in Figure 5, and was thought to render the comparisons between the experimental and control curves more significant.

The form of the average diurnal curve for each group was ascertained by averaging the times open or active for each of the 24 daily, one-hour periods of 15 consecutive days. These 24 values were converted further to the percentage of 60 minutes open or active. All phases of a possible tidal influence are represented in each of the values, and are thereby randomized since data for 15 days were used. In the cases in which the particular observational period was longer than 15 days, a 15-day period was arbitrarily selected, usually from the middle of the period.

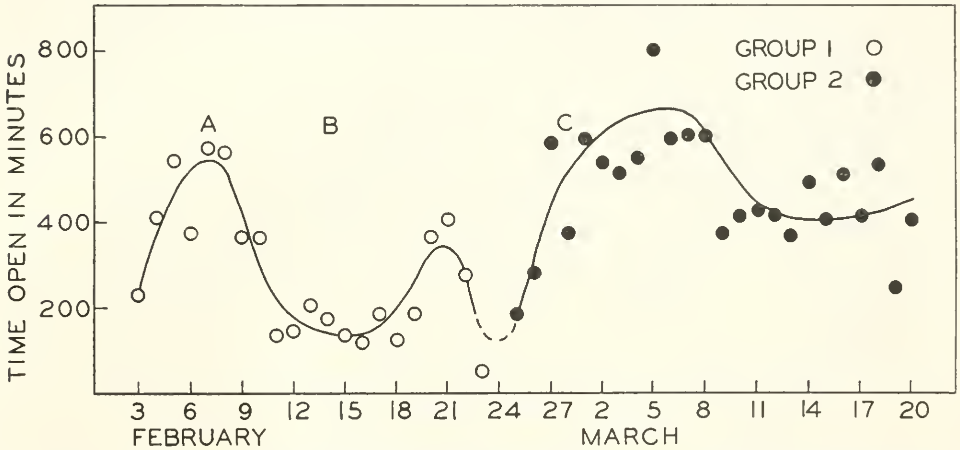

FIGURE 1. The long-term cycles of activity for group 1 (open circles) and group 2 (black circles) which were studied in Evanston, Ill. For explanation of A, B, and C, see text.

Conversely, the diurnal rhythm was rendered random to reveal the character of the tidal rhythm in the following manner. The values for activity of the 24 daily, one-hour periods for each of 15 successive days were moved backwards at an average rate of 50 minutes a day, *i.e.,* on succeeding days later one-hour periods became aligned with earlier ones. By this means any possible tidal cycles were kept in phase with one another in the vertical columns of data. By continuing this process, repeating with the data of the first 14 days until a total of 29 days had been included, every phase of a diurnal influence was moved across the whole 24-hour period. Again, each of the 24 values has been expressed as the percentage of 60 minutes active or open. In Figure 4 the percentage of each hour open was plotted against time in hours in such a manner that the first minimal point fell between the sixth and seventh hours. This procedure was obviously perfectly legitimate since the points on the abscissa merely indicated consecutive hourly periods, and do not represent actual times of any given day.

Results

1. The studies of animals maintained under constant conditions

A. The long-cycle rhythm

The average time open in minutes per day was determined for each group of quahogs. Long-cycle rhythms of activity were rendered clearly evident by plotting the time open (ordinate) against the date (abscissa). These cycles of activity for the four groups of animals are illustrated in Figures 1 and 2. It can be seen in the curve for groups 1 and 2 (Fig. 1) that major peaks of activity occur about 29 days apart and a minor peak occurs about half way between. The curve for group 3 (Fig. 2A) and for group 4 (Fig. 2B) show the minor peaks of a cycle with periods of lower activity on either side. It is also evident that the curve for group 3 can be superimposed on those for groups 1 and 2 from points A to C in Figure 1. Furthermore, the curve for group 4 simulates those for groups 1 and 2 from points B to C in Figure 1, and also that for group 3 from D to E in Figure 2A.

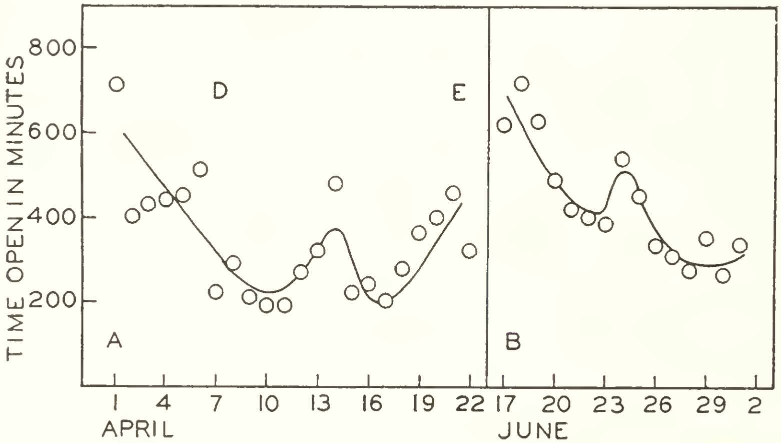

FIGURE 2. The long-term cycle of activity for group 3 (A) which was studied in Evanston, Ill., and that for group 4 (B) which was studied in Woods Hole, Mass. For explanation of D and E, see text.

It was thought that the clams in each of the first three groups were probably in synchrony with one another since they were reported to have been collected in the same area of the Virginia coast. Examination of the activity records of the individual animals of a single group supports this assumption. Within a group, all the individuals have their maxima and minima occurring within a particular 48-hour period. In group 4, the variation is even less. With the exception of one animal, all the latter showed their highest observed activity on June 17 and a secondary peak of activity on June 24. All the clams of this group had their lowest observed activity from about June 27 to June 29, correlated with the time of the semi-lunar low, low tides in Little Harbor.

B. The diurnal rhythms

The diurnal rhythms for the four groups of animals are shown in Figure 3. The average percentage of an hour open for each of the 24-hour periods was plotted against the time of day in Eastern Daylight Time, so that the cycles of the Evanston and Woods Hole groups could be compared directly.

Most evident in the curves of the diurnal rhythms are the facts that in all the groups, the maximal activity occurred in the afternoon, and that activity reached a low point during the morning hours. The afternoon maximum was recorded between 5 and 9 P.M., 3 and 4 P.M., 2 and 4 P.M., and 5 and 6 P.M., for groups 1, 2, 3, and 4, respectively. In groups 1, 2, and 3, the morning minimum occurred between 9 and 10 A.M. whereas in group 4, the time of minimal activity extended from 2 to 6 A.M.

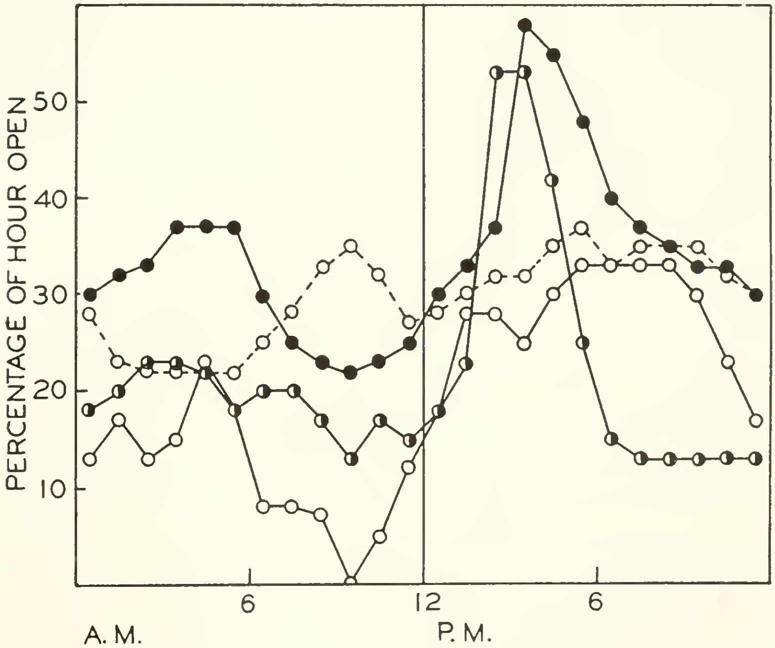

FIGURE 3. The diurnal rhythms of activity for the four groups of animals that were studied under constant conditions. Group 1 (open circles with solid lines), group 2 (black circles with solid lines), group 3 (black and open circles with solid lines), and group 4 (open circles with broken lines).

The diurnal rhythm of group 3 is striking in that a relatively long period of low activity is apparent between 7 P.M. and midnight whereas in groups 1, 2, and 4 the activity fell gradually from the afternoon highs during a comparable period of time. All groups of clams also had a minor peak of activity during the morning hours. In the first three groups these peaks were between 4 and 5 A.M., 3 and 6 A.M., and 2 and 4 A.M., respectively. The morning maximum in group 4 did not occur until 8 A.M. although the morning low occurred earlier, *i.e.*, between 2 and 6 A.M., than in the other groups. The minor peak of the morning occurred about 12 hours before the diurnal maximum in all the groups, except group 4.

C. The tidal rhythms

The tidal rhythms of activity, analyzed as described earlier, are illustrated in Figure 4. Two periods of lower activity are evident in each of these curves. These two periods of each curve are about 12 hours apart, thereby approximately reflecting the normal frequencies of the tides of the Atlantic coast. Also to be noted is the fact that the low points of the curve for group 4 occur near the time of low tides in the area of collection. This was the only group for which correlations could be made between actual tidal events and phases of the activity cycles. Since in rendering the diurnal cycles random in order to ascertain the tidal cycles, the successive tidal cycles were brought into phase with the tidal cycle of June 17, 1953, tidal conditions on this day are the ones which should be correlated with the resulting tidal curve. When the points were moved so that the first minimum came to lie between hours 6 and 7, the indicated times of the low tides were moved correspondingly.

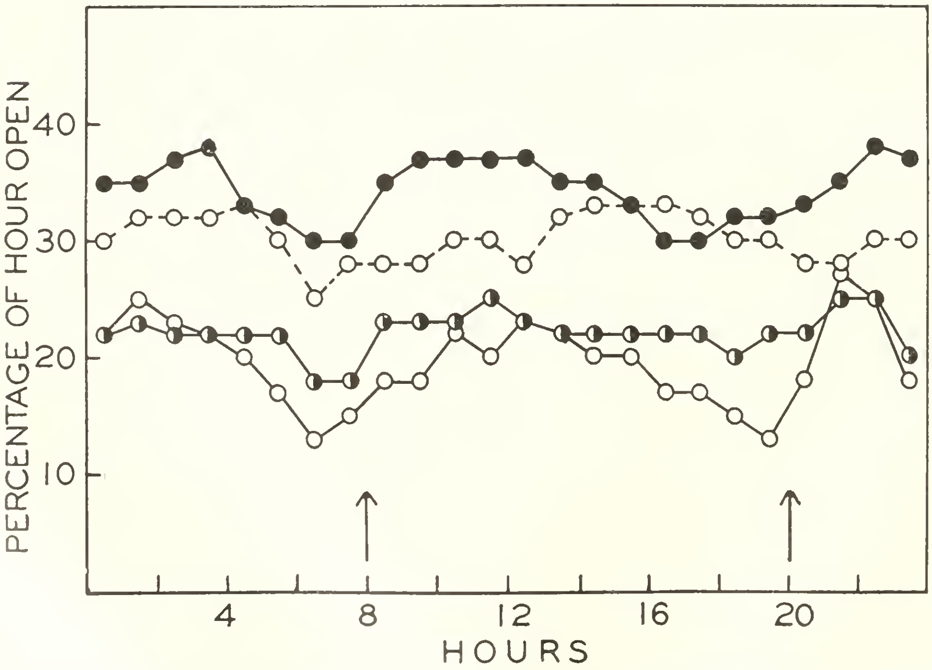

FIGURE 4. The tidal rhythms of activity for the four groups of animals that were studied under constant conditions. Group 1 (open circles with solid lines), group 2 (black circles with solid lines), group 3 (black and open circles with solid lines), and group 4 (open circles with broken lines). Note that the two periods of low activity for group 4 were near the times of low tide as indicated by the arrows.

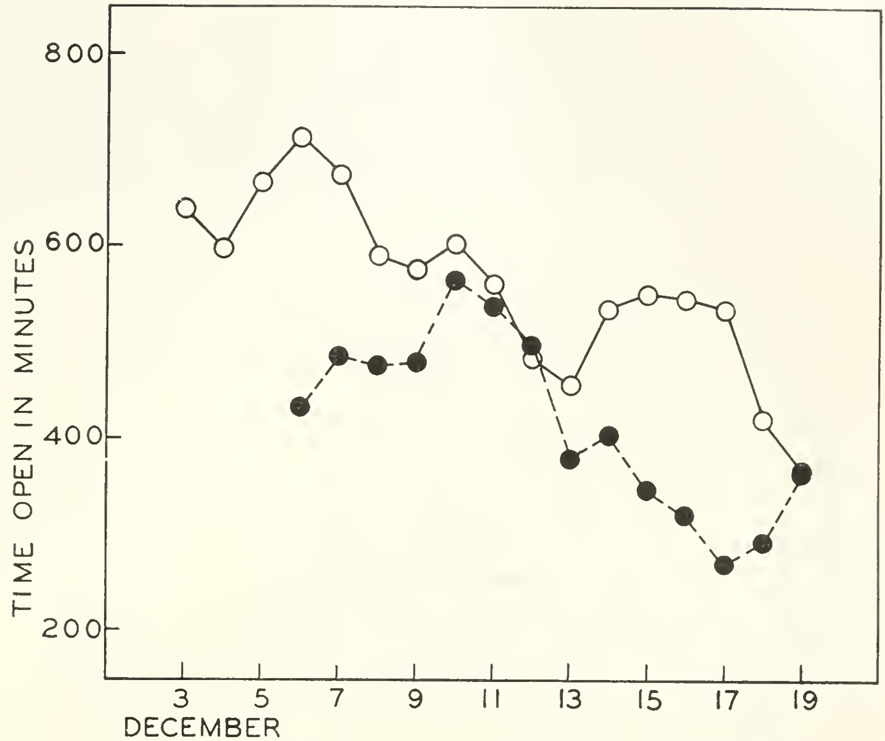

FIGURE 5. The long-term cycles of activity for the group of clams that was exposed to an illumination of 100 ft. c. by night and darkness by day for five consecutive days (broken lines) and for its control group that was maintained under constant laboratory conditions (solid lines).

The particular method used in determining the general character of the tidal cycle does not permit demonstrating precisely the expected 12.4-hour character of the tidal cycles. The curve for group 1 does show two minima occurring 13 hours apart while that for group 2 has low points 10 hours apart, group 3 11.5 hours apart, and group 4 14.5 hours apart. The average obtained, therefore, was 12.25 hours for the four series.

The amplitude of the four cycles also differs from one to another. That for group 1 has the greatest amplitude, an increase of 108% from lows to highs, while the other three increased only 27% to 32% from low points to high ones. Another interesting point evident in the curves of the tidal cycles is that in all of them one of the two periods of increased activity shows two peaks of relatively high activity separated by two to three hours of relatively low activity.

The shapes of the tidal curves for groups 1, 2, and 3 are similar to that illustrated for group 4 for which correlations with actual tidal phases could be made. Therefore, it would be reasonable to predict tidal events in the habitats of groups 1, 2, and 3, since the phases of the persistent tidal cycle probably always bear corresponding relationships to the times of tides in the native habitat.

II. The effects of reversed illumination on the activity cycles

The effects of reversed illumination, *i.e.*, 100 ft. c. from 8 P.M. to 8 A.M. and darkness from 8 A.M. to 8 P.M. for five days, on the long-cycle activity are presented in Figure 5. By comparing the curves for the experimental group and its control, it appears that the illumination changes effected an over-all lower activity for the treated group. The average time open per day for the 20-day observational period is 574 minutes for the controls and only 432 minutes, or about 25% less, for the experimental group.

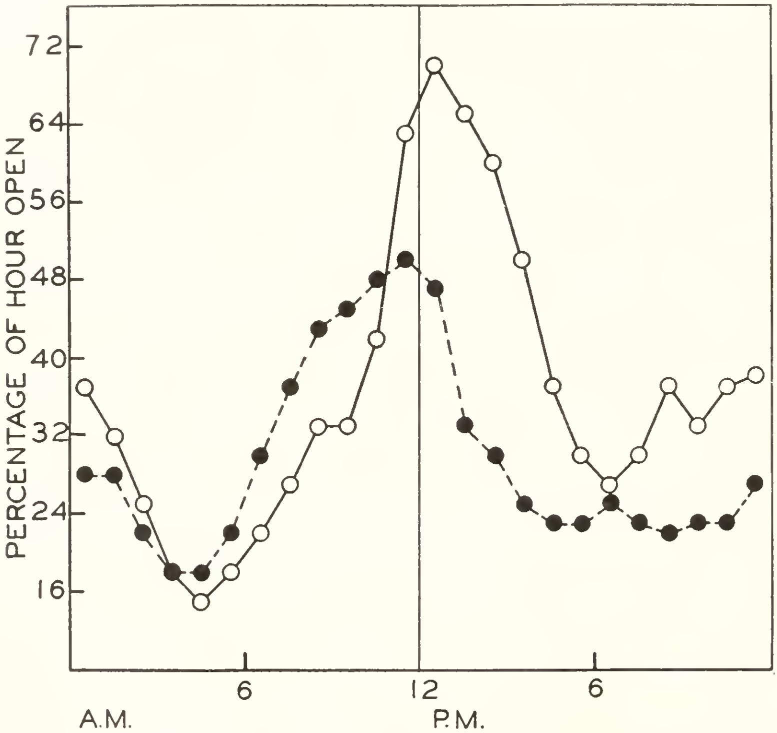

FIGURE 6. The diurnal cycles of activity for the group of clams that was exposed to reversed illumination (broken lines) and for its control (solid lines). The cycle for the experimental group appears to have been shifted one hour to the left or earlier when compared to that for the control group.

A change in the temporal relationships of the curve for the experimental group is also evident in Figure 5. This shift can be seen best by comparing the high point of December 6 on the control curve with a comparable high on December 10 on the experimental one. Low points also occurred four days apart, *i.e.*, on December 13 for the controls and on December 17 for the treated animals.

The general form of the long-term cycle of the control group, as well as that of the experimental group, was seen to be similar to the general form of the long-term cycles of activity that were found for the first four groups of quahogs (Figs. 1 and 2). It is probable that the portion of the cycle from December 13 through

December 19 of the control curve (Fig. 5) represents a minor peak which corresponds to that of groups 1 and 2 between February 18 and 24 (Fig. 1), to that of group 3 from April 11 through 17 (Fig. 2A), and that of group 4 from June 22 through 28 (Fig. 2B). It seems highly probable that the points on the control curve (Fig. 5) prior to December 13 represent the descent from a major peak. If this is true, a major peak would have occurred 15 days earlier or about December 1. A high value recorded for December 1 supports this view. However, this was the first day that the quahogs were in the laboratory and therefore the value was not incorporated in the figure.

The diurnal rhythms for the two December groups were strikingly similar to one another (Fig. 6). Again, the greater activity of the control animals is evident in this figure, and it is seen that the larger part of this higher activity is contributed by a greater diurnal maximum. The curve for the experimental group shows merely a slight shift in temporal characteristics when it is compared with the control cycle. The diurnal maximum for the experimental group occurred one hour earlier than that for the controls, and the diurnal minimum lasted for two hours (3 to 5 A.M.) in this former group as opposed to one hour (4 to 5 A.M.) in the latter, similarly indicating a small shift to an earlier time.

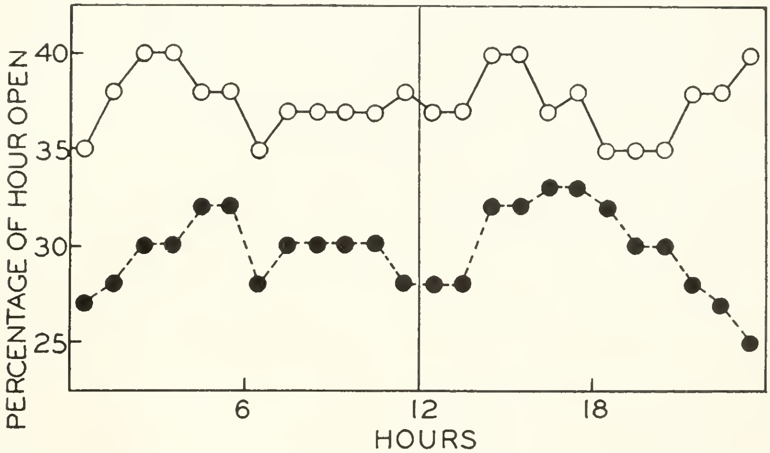

FIGURE 7. The tidal cycles of activity for the group of animals exposed to reversed illumination (broken line) and for the group of its controls (solid lines).

A comparison of the 24-hour cycles of the two December groups with those of the four groups that were studied earlier in the year (Fig. 3) shows that there are certain likenesses in form, although the temporal aspects of the curves differ. The most obvious difference is that the diurnal maximum for the December groups occurred just before and just after noon whereas the other curves had maxima later in the afternoon. The morning minimum for the December groups was also earlier, *i.e.,* between 3 and 5 A.M., as were the minor peaks of the morning which occurred immediately after midnight. The period between the minor and major peaks, however, was again 12 hours as in groups 1, 2, and 3.

The tidal cycles of the December groups (Fig. 7) are also similar to those of the earlier groups (Fig. 4). The two minima are separated by about 12 hours and one of the prolonged periods of increased activity is characterized by one or two hours of relatively low activity between periods of higher activity.

It is clearly apparent that the tidal cycle for the group of clams that was exposed to five periods of reversed illumination was shifted in time relative to that for the control group (Fig. 7). Each of the two minima of the experimental curve occurred about five hours later than the comparable lows of the control group. Again, in this figure, the lower average activity of the experimental animals is evident.

Discussion

The evidence at hand supports the assumption that the long-cycle rhythms illustrated in Figures 1, 2, and 5 are actually lunar cycles or parts thereof. The curve drawn between points contributed by groups 1 and 2 shows almost exact lunar periodicity; the maxima are 29 days apart and the minor peak occurs halfway between the two major ones. The time to minimal activity following the minor peak of February 21 and preceding the minor peak of March 7 (Fig. 1) appears shorter than the comparable times for groups 3 and 4 (Fig. 2). Since, however, the overall activity for the clams of group 2 was greater than that for those in group 1, it is suggested that the point for February 25 really constitutes that of minimal activity. On this basis, the time for passage from the lesser maximum to the minimum would be three to four days in every instance.

It was first thought that the clams of group 3 might have been collected from the same region of Chesapeake Bay as those of groups 1 and 2. If these three groups of animals were in tidal, and consequently in lunar synchrony a major peak of activity would be expected to have occurred about April 5 and a minor one about April 20. It is seen from inspection of Figure 2A that this is not the case, but rather an apparent decline from high activity characterizes the cycle about April 5 and a minor peak appears on April 14.

Several explanations for this diversity are plausible. It is very probable that the animals of group 3 had been collected from an area of Chesapeake Bay in which the times of tides differ from those in the region from which the clams of groups 1 and 2 had been obtained. The times of tides in different parts of Chesapeake Bay vary quite considerably because of the topography of this area. Secondly, it is possible that all these animals were collected in the same region but that the rhythms for group 3 were put out of phase by light, temperature, or some other stimulus to which the clams were subjected during shipment from the Atlantic coast to Evanston.

No correlations between the phases of the tidal cycle for group 3 and actual tidal events could be made. Therefore, it is impossible to know whether or not the phases of this tidal rhythm were shifted. Also, the diurnal cycle of activity for group 3 did not differ basically from those for groups 1 and 2. Unpublished observations suggest that for fiddler crabs, the persistent diurnal and tidal rhythms, the summation of which determines the phases of the long-cycle rhythms, are shifted together by temperatures that approach freezing. This is also true of the cycles for fiddler crabs that are shifted by exposure to an illumination of high intensity (Brown, Fingerman, Sandeen and Webb, 1953). Nevertheless, the data on the effects of changes in illumination on the rhythms for quahogs, reported in the present paper, appear to indicate that the diurnal and tidal cycles were affected differentially by this treatment.

It was seen that the period of minimal activity for group 4 was correlated roughly with the semi-lunar low, low tides which occurred just after the full moon of June 26. It was therefore thought that the long-cycle rhythms for the other groups also might be correlated directly with phases of the moon. This does not seem to be the case, however, since a full moon occurred on February 28, 1953, or about seven days after a minor peak for group 1, on March 28, 1953, or about two weeks before a minor peak for group 3, on June 26, or two days after a minor peak for group 4, and on December 20, or about four days after a minor peak for the group of clams that served as controls in the experiment on effects of reversed illumination. These facts suggest that it is the time of the local tides which determines the phase relationships of the semi-lunar and lunar cycles rather than a more direct lunar influence. This hypothesis is supported by results of the study of the tidal rhythms of color change for *Uca* (Brown, Fingerman, Sandeen and Webb, 1953), which demonstrated that the cycles of crabs that inhabit areas, the tidal times of which differed by about four hours although located in the same geographical region, were correspondingly four hours out of phase with one another. Therefore, the phases of the long-term cycles for quahogs that live in areas of Chesapeake Bay which differ in times of tides would likewise differ from one another, although all the animals would be exposed to the same phases of the moon at the same times.

The similarity of the curve for the tidal cycle for group 4 to those for groups 1, 2, 3, and the group that served as controls in the illumination experiment, and the correlation of the phases of the rhythm of group 4 with actual tidal events, suggest strongly that this same correlation could be made for the latter groups if the exact data on tides were available. All the groups showed diurnal cycles of activity. The investigation by Brown, Fingerman, Sandeen and Webb (1953) proved conclusively that the presence of both persistent tidal and diurnal rhythms in the same animal produces a persistent semi-lunar rhythm since the tidal cycle is in the same relationship with the diurnal cycle at 14.8-day intervals.

If the effect of alternate tidal cycles on metabolism or activity differs quantitatively or qualitatively from that of the intervening tidal cycles, the tidal cycles would be in a similar relationship with the diurnal cycle only at 29.5-day intervals, or lunar ones. This situation appears to be true for the long-term rhythm of the quahogs, since the alternate semi-lunar maxima are larger than the intervening ones. The activity data for only 15 consecutive days were used in the analysis of each of the tidal rhythms. Therefore, when the data for the first 14 days were repeated in the analysis, values which included the effects of both of the daily tides were averaged, and any difference between them was obscured. The tidal rhythm of O_2-consumption for the snail, *Littorina littorea* (Sandeen *et al.,* 1954), was analyzed in the same manner as those reported in the present paper, and it appears to show more clearly than those for the groups of quahogs that the effects of the alternate tidal cycles are not equivalent.

At present, the diurnal cycle of activity of the quahogs cannot be characterized precisely. As has been seen, the activity generally reached a maximum during the afternoon hours and minimal activity occurred during the morning hours. It is possible that the differences that were seen among the diurnal rhythms for the various groups reflect adaptations of the diurnal activity to the varying lengths of daylight throughout the year. The cycles for groups that were observed during March, April, June, and December show indications that during periods of shorter day-length the greater activity of the clams occurred earlier in the day than it did during the times of the year when days are longer. Activities for groups 2 and 3 which were observed in March and April declined after 4 P.M. Group 4, which was studied in June, showed an hour of maximal activity between 5 and 6 P.M. Correspondingly, the diurnal cycle for the controls for the animals that were exposed to light and dark and were observed in December reached a maximum just after 12 noon. The activity decreased rather rapidly after this time. If this assumption is correct, it is seen that the rhythm for group 1 is not in line with those of the other groups. Group 1 was observed in February at which time daylight hours are fewer than in March and April in the northern hemisphere, but its period of maximal activity extended from 5 P.M. until 9 P.M.

The periods of minimal activity for the different groups of animals also show some correlation with the day-length that existed at the times of observation. For groups 1, 2, and 3, which were observed during the early spring, the diurnal lows were recorded between 9 and 10 A.M., at which times the groups were active 0%, 22%, and 13% of the hour. The period of low activity for group 4, studied in June, ended shortly after 6 A.M., and at this time these animals were active 25% of each hour period. The cycle for the December control group does not fit into this series. Here the morning minimum was reached between 4 and 5 A.M. when the clams were open 16% of the hour. However, these varying diurnal rhythms may be indications of an annual cycle, and yet not reflect day-length directly.

The data for the persistent diurnal rhythms of O_2-consumption for marine snails (Sandeen *et al.*, 1954) and for fiddler crabs (Brown, Bennett and Webb, 1954) suggest that the diurnal patterns may change slightly in accordance with day-length. In these cases, as in the present report, the animals were maintained under constant laboratory conditions. Brown and Stephens (1951) have shown that the day-phase of the diurnal cycle of color change for *Uca* can be influenced by the length of the daily photoperiod to which series of crabs are exposed. The changes in this cycle, once induced, persisted after the animals were placed in constant darkness.

It is interesting to note, once more, that an earlier study of *Venus mercenaria* in its natural environment did not yield any positive suggestions of rhythmic activity (Loosanoff, 1939). It is possible that in an organism's natural habitat, characteristics of the basic persistent rhythms, *e.g.*, frequency, form, and amplitude, are masked or overshadowed by varying external stimuli such as light, temperature, and movements of the water. A suggestion that this may be true is found in the tremendous increase in the amplitude of the diurnal rhythm of color change in *Uca pugnax* after the crabs have been brought from the field and maintained in constant darkness (see Figure 10, Brown, Fingerman, Sandeen and Webb, 1953).

As is true for the persistent rhythms for fiddler crabs, those for *Venus* can be modified to some degree by exposing the animals to alternating periods of darkness and illumination. It has been shown that five consecutive days (8 A.M. to 8 P.M.) of darkness with five nights (8 P.M. to 8 A.M.) of illumination of 100 ft. c. effected changes in the diurnal, tidal, and long-cycle rhythms of activity for a group of clams. Whether or not the apparent shift of the diurnal cycle is significant is difficult to state. As discussed previously, the diurnal rhythms for the different groups of animals that were observed varied to the extent that a precise characterization of this rhythm could not be made. Furthermore, it is not known whether these variations reflected adaptations to day-length. Therefore, the diurnal cycles of activity illustrated for the experimental group and its control (Fig. 6) may be in phase with one another. However, the moving of both the morning minimum and the afternoon maximum for the experimental group by about the same amount of time and in the same direction, *i.e.,* one hour earlier than the comparable points for the control group, seems to indicate that the phases of this cycle were shifted.

The effects of five consecutive days of darkness and five nights of illumination on the tidal and long-term cycles of activity can be discerned more readily. The tidal rhythm for the experimental group seems to be shifted so that the low points occurred five hours later than the comparable points for the control group (Fig. 7). In other words, the tidal cycle of the experimental animals is about five days ahead of the control one. The phases of the long-term cycle for the experimental clams occurred four days after those of the controls (Fig. 5). Since the long-cycle rhythm is the result of the moving of the tidal influence across the diurnal cycle at the rate of about 50 minutes per day, or so that the tidal effect occurs 50 minutes later on each successively later day, the apparent five-hour shift of the tidal rhythm could not have effected a shift of the long-cycle rhythm such that its phases occurred four days late. If the experimental tidal rhythm had been shifted so that on a hypothetical day the low point of the cycle, which probably correlates with the time of low tide in the natural habitat of the clams, occurred between 11 P.M. and midnight and the comparable low point for the control group occurred five hours earlier, or between 6 and 7 P.M. on the same day, the resulting long-term cycle for the experimental animals would be moved so that its phases would occur earlier rather than later than those of the cycle for the control group.

In order that the phases of the long-cycle rhythm for the experimental group be four days late when compared to those of the control group, the tidal cycle for the former would have had to be shifted so that a low point occurred about four to five hours earlier than, or that the tidal cycle of the experimental group was four to five days behind, that of the control group. As has been seen, this was not the case (Fig. 7). However, when a particular point of the experimental cycle which is the result of a tidal influence appears to be five hours late, or about four to five days ahead, it may in reality be nearly 20 hours early, since a particular tide recurs about every 24.8 hours. If this situation exists, the phases of the long-cycle rhythm for the experimental animals would be expected to occur about 25 days before those of the control cycle (Fig. 8). The facts that a particular tide occurs about 50 minutes later on each successively later day, and that the long-cycle rhythm is repeated about every 29 days, account for this relationship.

Several hypotheses may be offered to explain these shifts. 1) The first dark-to-light or the first light-to-dark change may have been the stimulus that effected the movement of the phases of the tidal cycle for the experimental group about 20 hours backwards, and consequently, the phases of the long-cycle rhythm 25 days ahead of those for the control clams. 2) Possibly, the effects of the changes of illumination or the periods of high illumination or darkness were cumulative, and thereby caused the shifts that have been discussed. On the basis of the results of previous investigations it is probable that the changes from darkness to light and the light period were the effective factors. Webb (1950) found that the phases of the diurnal rhythm of color change for fiddler crabs that were maintained in constant darkness were shifted by single illumination periods of varying lengths. The direction and the amount of shift were dependent on the time of day at which the animals were exposed to an illumination of 90 ft. c. A more recent paper (Brown, Fingerman and Hines, 1954) presents an hypothesis to account for the graded series of shifts of the diurnal cycle of color change for *Uca*. These graded shifts in the phases of the cycle were noted when series of crabs were exposed to various higher illuminations by night and various lower ones by day. Two factors appeared to operate in the altering of the phases: 1) the strength of the light-increase stimulus, and 2) the brightness of the higher illumination to which the animals were exposed by night.

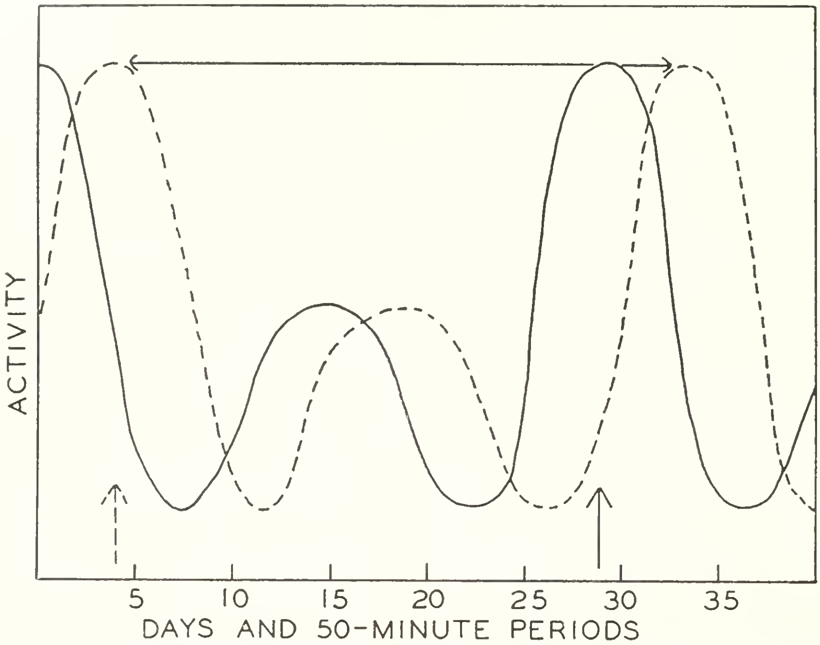

FIGURE 8. An illustration to show the effects of shifts of the tidal cycle of an experimental group of clams on the long-term rhythm of activity. The broken line curve is that of the experimentals and the solid line one is that of the controls. The broken line arrow indicates the time of low tide for the experimental group and the solid line one for the control group. The horizontal arrows indicate the directions and amounts of shifts possible. (For discussion, see text.)

Exposures of fiddler crabs to several cycles of altered illumination operate to shift the tidal rhythms of color change as well as the diurnal cycles (Brown, Fingerman, Sandeen and Webb, 1953). Here the phases of the tidal and the diurnal rhythms were moved in the same direction and by the same number of hours. In this case, the temporal relationship which existed between the two cycles at the times of exposure of the crabs was maintained. In the present study, the data lead to the assumption that the tidal rhythm for the experimental group of quahogs was shifted so that its phases occurred about 20 hours earlier than those for the animals that were exposed to constant low illumination. The shift of the diurnal rhythm was, at most, only one hour in the same direction. The diurnal cycle could have been shifted by a number of days, however, and this could not have been ascertained. For example, if each of the five alternating light cycles had shifted the diurnal rhythm backwards by five hours, it would appear to have been set backwards only one hour. Meanwhile, if the tidal influence had each day moved forward 50 minutes over the diurnal cycle while each day the diurnal cycle was set backwards five hours, the phases of the tidal cycle would be in effect set backwards about 20 hours during the five days. This interpretation is so clearly compatible with the second hypothesis advanced earlier that its likelihood is great.

From the results of the present and earlier investigations, it is evident that light stimuli are effective in setting phases of both persistent diurnal and tidal rhythms, and consequently the semi-lunar and lunar ones. Two other investigators recently have discussed the problem of setting the phases of persistent cycles. Brett (1954) saw that a light flash of only one minute was sufficient to set the phases of the diurnal rhythm of emergence for *Drosophila*. The tidal movements of the water seem to participate in determining the phases of the tidal rhythm for *Mytilus* (Rao, 1954).

The question remains: are these persistent rhythms endogenous or exogenous? If they are endogenous, or dependent upon basic physiological mechanisms, the various experimental procedures which shift the phases of the cycles have actually altered the "internal clocks." On the other hand, if the rhythms are controlled by exogenous forces, the manifestations of the rhythms have merely been moved from their normal relationships with the basic cycles when shifts are apparent. The present study contributes no evidence concerning this problem, but studies planned for the near future should provide an answer.

Precise rhythms of activity such as those discussed in the present paper are seemingly adaptive for this species of clam. Loosanoff (1937) states that the gonads of adult *Venus mercenaria* inhabiting Long Island Sound appear to contain ripe sperm or ova throughout the greater part of the year. Yet, spawning does not occur in the majority of animals until August. A certain critical water temperature has been thought to be the stimulus for spawning (Loosanoff, 1937). Although temperature may be a limiting factor it seems more probable that the presence of persistent lunar rhythms, resulting from persisting diurnal and tidal ones in these clams, may eventually be shown to be the immediate regulator of spawning activities. Such a mechanism would provide more precision than could be obtained by the very slow, gradual temperature change of the water.

The greater activity of the clams during the day than during the night may be adaptively correlated with the availability of food. Plankton populations, which supply much of the food of this species, are known to undergo diurnal rhythms of migration. In general, the greatest density of plankters near the surface is found during the hours of the night, and conversely, the greatest density at greater depths occurs during daylight (Russell, 1927).

SUMMARY

1. Continuous kymograph recordings of the opening and closing of the valves of common quahogs, *Venus mercenaria*, secured from the Virginia coast and from

Woods Hole, Massachusetts were made during February, March, April, June, and December, 1953.

2. The activity records of these clams maintained under constant laboratory conditions show a persisting diurnal rhythm with maxima and minima occurring in the afternoon and early morning, respectively. There is some evidence that the pattern of the diurnal cycle varies with the time of year.

3. A persisting tidal rhythm is also evident. In the group of animals for which correlations with actual tidal phases could be made, it was seen that the times of minimal activity in this cycle correspond closely with the times of low tide in the area from which the animals were collected.

4. The summation of these two cycles, diurnal and tidal, is responsible for an observed lunar cycle. The phases of the lunar cycle are probably set directly by the tides to which the animals are exposed rather than by any direct lunar influence.

5. The rhythms of a group of clams that was exposed to darkness during the day (8 A.M. to 8 P.M.) and to an illumination of 100 ft. c. during the night (8 P.M. to 8 A.M.) for five consecutive 24-hour periods show shifts in their cycles when compared to those of control animals. The shifts are such that the phases of the diurnal rhythm occurred one hour earlier than those of the controls; the phases of the tidal cycle occurred about 20 hours earlier than those of the controls; and the phases of the long-cycle rhythm were, in consequence, 25 days ahead of those of the controls.

6. The manner in which these shifts of the phases of the cycles are related is discussed and illustrated.

7. Adaptive significances of these persistent rhythms in these clams are suggested.

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ON THE RESPIRATION IN SCALLOPS (LAMELLIBRANCHIATA)[1]

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The only data in the literature on the respiration of scallops seem to be those of Spärck (1936; *cf.* also Thorson, 1936), who determined the oxygen uptake in *Pecten groenlandicus* (arctic), *P. varius* (boreal-mediterranean), and *P. flexuosus* (mediterranean) in nearly air-saturated water. This author found that the resting metabolism of the scallops, and other bivalves which have a marked capacity for rapid swimming locomotion, is considerably higher than that of more sedentary types.

The present paper reports on a study of the respiration in the deep sea scallop and the bay scallop, both of common occurrence and of considerable economic importance along certain areas of the Atlantic coast of North America. The oxygen consumption of resting (non-swimming) individuals was determined over a range of concentrations of oxygen of about the air-saturation value to close to zero. In addition determinations were made, in aerated water, of the oxygen utilization or the percentage of oxygen removed from the inspired water during its passage through mantle cavity and gills.

MATERIAL AND METHOD OF OXYGEN ANALYSIS

The deep sea scallops (*Pecten grandis* Solander) were taken from Cape Cod Bay, at a depth of about 12 m., the bay scallops (*Pecten irradians* Lamarck) from a shallow salt water pond near Woods Hole. All animals were kept in the laboratory in tanks with running sea water (salinity 31–32.5‰). For the experiments, which were carried out during June and July, 1952, and from September–November, 1953, only specimens that were in apparent good health, showing a wealth of actively moving, extended tentacles and production of faecal material, were used.

Analyses of dissolved oxygen were made by the Winkler method on samples of about one ml. (van Dam, 1935a).

TECHNIQUES OF MEASUREMENTS OF O_2-CONSUMPTION

(a) Continuous measurements of O_2-consumption in water approximately saturated with air were performed on single individuals of the sea scallop by means of a volumetric respirometer technique as described by Scholander (1949). In this method the oxygen consumed is directly read on a syringe from which oxygen is introduced into the system at the rate at which it is removed by the animal.

A plastic box of about 900 ml. capacity, immersed in a water bath, was used as a respiration chamber. It was filled with about 850 ml. of sea water which had been filtered through cotton or glass wool. Prior to the actual experiments the animals were kept for one hour or longer in the respirometer running open to the

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[1] Contribution no. 705 from the Woods Hole Oceanographic Institution.

air. Runs without an animal in the water showed a blank oxygen consumption of, on the average, about 0.2 ml. O_2/hr. (Fig. 5). The cause of these rather high blank values is not known. The calculated Q_{O_2}-values (O_2-consumption per kg. and hour) given in Figure 4 are STP- and blank-corrected. In a number of tests initial and final O_2-content and approximate pH (Hellige comparator) of the water in the respirometer were determined, showing that, as a rule, the O_2-content remained practically constant. In a few cases a decrease of 0.1–0.3 ml. O_2/L. was observed, and the Q_{O_2}-values were corrected accordingly. Although in this method the CO_2 produced is continuously being removed, the pH of the water during the tests usually decreased from about 8 to about 7.2–7.6. This, however, as can be seen from Figure 5, had no apparent effect on the rate of oxygen uptake. The same holds for the experiments with the closed respiratory chamber technique described below, in which all excreta, CO_2 included, remained in the water.

(b) To determine the critical O_2-tension, measurements of O_2-consumption were made in closed plastic respiration chambers of 1–3 L. capacity (Fig. 1), completely filled with water, in which the concentration of oxygen decreased progressively due to the respiration of the animal.

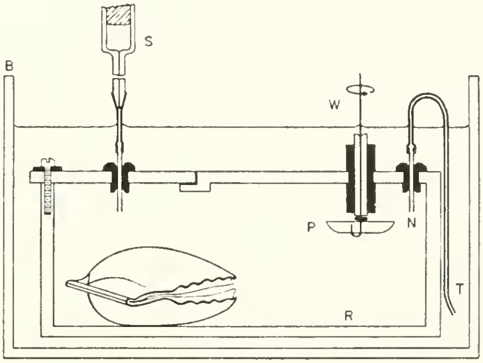

FIGURE 1. Diagram of closed respiratory chamber (R). W, P: stainless steel drive shaft and plastic paddle. T, N: small bore plastic tubing and needle through which water from water-bath B enters R when water samples for O_2-analysis are taken with syringe S.

Blank runs (Fig. 6) showed that in the absence of an experimental animal the O_2-content of the water in the respirometer was practically constant at any O_2-tension. Control tests showed that the Winkler procedure, as such, was not interfered with by metabolites in water in which animals had been confined for several hours.

Prior to the O_2-uptake measurements the animals were scrubbed clean thoroughly and acclimated to the experimental temperatures for one day or longer. To determine the wet weight of the soft tissues, the animal was opened by severing the adductor muscle. Then all soft tissues were taken out of the shells, drained for about 5 minutes, dried at the outside on filter paper and then weighed. Length and weight of the animals used are given in Table I.

TECHNIQUE OF DETERMINING THE OXYGEN UTILIZATION

The sea scallops were lying freely in a normal position, *i.e.*, on the right valve, on the bottom of an all-glass aquarium. Samples of the inhalant water current were taken with a syringe, the tip of which was placed near the pallial opening opposite the middle of the hinge. Simultaneously, samples of the exhalant current were taken, either directly by means of a syringe to which a fine glass tip, bent at right angles, was attached (animals no. 1 and 5, 18 samples), or by means of a siphon as indicated in Figure 2 (animal no. 6, 12 samples). Glass tip or siphon were introduced about 5 mm. into the cloacal chamber. Water from the cloacal cavity was allowed to pass through the siphon (capacity 7 ml.) for about one hour, at a rate of 1.5 ml./min., before the first samples were taken. There were no signs that the presence of tip or siphon, which, as a rule, did not touch the animal's tissues, interfered with the normal vigorous propulsion of water. Slowness of sampling guaranteed that the sample was not contaminated with outside water. A continuous flow of fresh sea water combined with an intensive stirring by means of big bubbles

TABLE I

*Length and weight of experimental animals**

Specimen no.	Number of test in which specimen was used		Length (mm.)	Wet meat (gms.)	Total wet weight (gms.)
	Test no.	Fig. no.			
		Sea Scallop			
1	—	—	146	—	—
2	1–4	5	59	9	25.0
3	7, 8	5	63	10.5	29.5
4	5, 6	5	69	13.5	40.0
5	9–13	5	81	23.0	88.0
6	14, 15	5	75	18.0	50.0
7	7, 8	6	73	17.8	49.0
8	1, 2	6	101	34.5	110
9	3, 6, 9	6	139	78.8	294
11	11	6	130	60.3	235
		Bay Scallop			
1	—	—	56	6.4	31.9
2	—	—	56	7.0	33.5
3	4, 10	6	61	10.2	38.1
5	5, 13	6	63	12.5	39.0
6	5, 12	6	59	10.5	33.8

* The length given here denotes the straight-line distance between the middle of the hinge (p) and a point at the margin of the upper shell opposite p. Due to circumstances, the wet meat weight of the sea scallops 2, 3, 4, 5 and 6 was not determined directly but was taken from a graphic representation of data, collected by Mr. J. A. Posgay, relating length and weight of wet meat in over one hundred specimens.

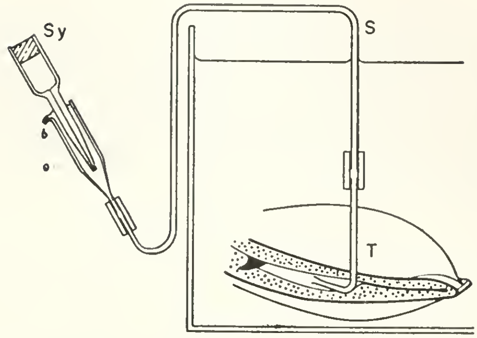

FIGURE 2. Sampling from the exhalant water current in the sea scallop by means of a cloacal siphon. From the continuously overflowing water, samples of about one ml. are taken with a syringe pipette (Sy). The lower end of the glass tube T is about 5 mm. inside the (white) cloacal chamber. Stippled: mantle. Black: tip of gills. Diagram.

of air kept the O_2-content of the water in the experimental aquarium uniform over prolonged periods of time.

In the earlier studies of the oxygen utilization in Lamellibranchia, made on siphonate forms, it has been assumed that, due to turbulent mixing in the relatively long and narrow siphon, the oxygen in the water leaving the exhalant aperture is fairly uniformly distributed. In the scallops, however, as in many other species, a siphon is lacking and the cloacal chamber communicates with the exterior through a normally widely opened slit. To determine whether appreciable O_2-gradients occur in the broad ribbon of water escaping from this slit, in the bay scallop two or three samples were taken simultaneously at different spots. The technique used is indicated in Figure 3.

As a rule the introduction of the needles into the cloacal cavity disturbed the ventilation only during the first few minutes and in several cases was accomplished

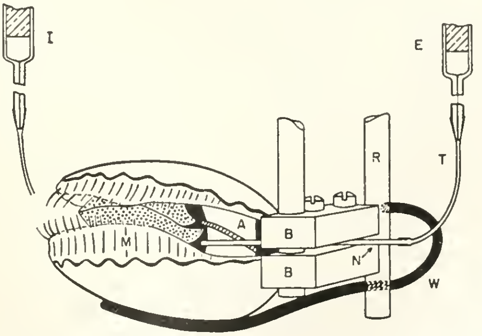

FIGURE 3. Sampling from the exhalant water current in the bay scallop by means of 2 or 3 needles (N), held, in a parallel fashion, 3–10 mm. apart, in a plastic block (B). The needles, of which only one is shown, protrude ½–1½ cm. into the cloacal chamber (black), and are connected with a sampling syringe (E). The scallop is paraffined onto a piece of wire (W), held in a plastic rod (R). I: One of the two syringes, sampling simultaneously, at different spots, from inhalant current. M: mantle. Stippled: gills. A: adductor muscle. Hatched: intestine, opening, at the left hand side, into the cloacal chamber (black). Diagram.

without any visible reaction on part of the animal. Moreover, sampling was not begun until after at least one hour had elapsed. On several occasions the needles were left in position overnight. By manipulating two syringes at a time and changing over from one pair to another every 10 seconds, simultaneity of sampling was accomplished to a fair degree. Sampling was interrupted when an animal would interrupt the pumping and was only resumed a couple of minutes after normal ventilation had begun again.

Discussion of Results

(a) Oxygen consumption in approximately air-saturated water

In Figure 4 the values for the oxygen consumption of the sea and bay scallop obtained in the present study (dots and crosses, respectively) are compared with data on the oxygen uptake of various other marine Lamellibranchia selected from the literature. The latter were grouped together and averaged according to geographical distribution (arctic, boreal, and mediterranean) and locomotory faculties (swimming versus non-swimming) of the animals. The relation between these

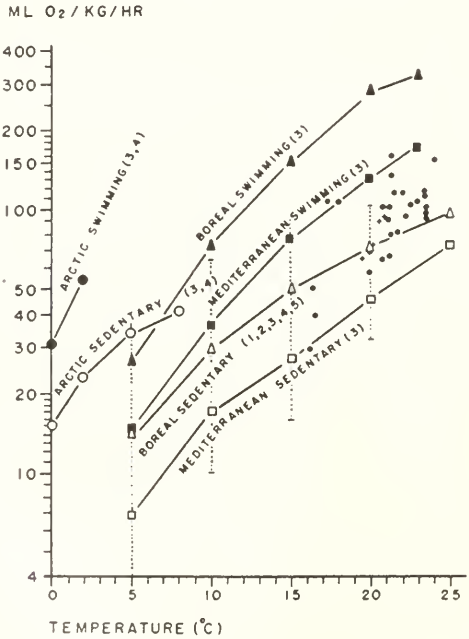

FIGURE 4. Oxygen consumption of resting specimens of bay and sea scallop studied in the present paper (crosses and dots, respectively) compared with the oxygen uptake of other marine Lamellibranchia, selected from the literature and grouped together according to geographical distribution (arctic, boreal and mediterranean species) and according to locomotory faculties (swimming or more or less sedentary species). On the curves, the points represent the arithmetical mean of all Q_{O_2}-values available for a given temperature. The figures in brackets refer to the source in the literature from which the data, used to compute these mean values, were taken, as follows: 1 = Bruce, 1926; 2 = Thamdrup, 1935; 3 = Spärck, 1936; 4 = Thorson, 1936; 5 = Whedon and Sommer, 1937. Vertical, stippled, lines: spread of data in the group of the boreal sedentary species.

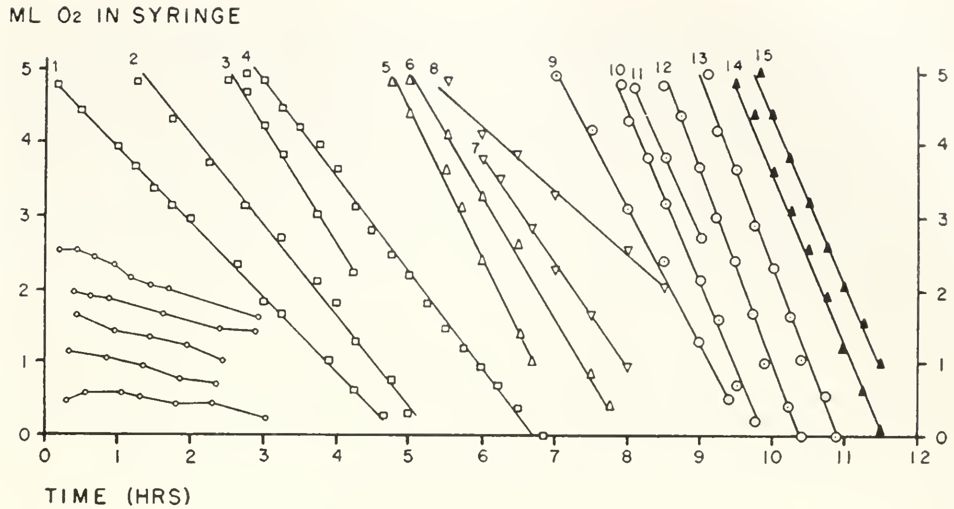

FIGURE 5. Rate of oxygen uptake in the sea scallop in approximately air-saturated water. Ordinate: amount of oxygen (ml.) in the measuring syringe. Abscissa: time (hrs.). Numbering of tests as in Table I. Small circles: blank runs.

factors and the metabolic rate in marine Lamellibranchia has been dealt with already by Spärck (1936). The following data (adult specimens, outside the spawning season) were used: Spärck (1936): 9 arctic, 12 boreal, and 11 mediterranean species; Thorson (1936): 11 arctic species; Thamdrup (1935): 3 boreal species; Bruce (1926): one boreal species; and Whedon and Sommer (1937): one boreal species. The figure indicates that the O_2-consumption in the bay scallop is essentially the same as that in the sea scallop. Over the range of temperatures investigated (16–24° C.), the O_2-uptake in these species is somewhat intermediate in position between the O_2-uptake of mediterranean scallops and that of more sedentary bivalves from that area and thus is about the same as the O_2-uptake of the average boreal non-swimming lamellibranch. In using the curves in Figure 4 as a basis for comparison, it must be borne in mind, however, that they give a greatly simplified picture inasmuch as the data, from which they were computed, show a very considerable spread in most cases. The spread in the Q_{O_2}-values of the different species of lamellibranchs in the boreal-sedentary group, is indicated in the figure (stippled lines). Not enough data are available to establish, in the bay and sea scallop, a relation between Q_{O_2} and season or size, or to estimate Q_{10}.

For several species of bivalve molluscs, lengthy periods of stoppage of the water propulsion have been reported in the literature. To the list of species given by van Dam (1938, p. 123; *cf.* also Verwey 1952, pp. 189–193) can be added *Venus mercenaria* (Collip, 1921; Chipman and Galtsoff, 1949) and *Hyridella australis* (Hiscock, 1950; 1953). Persistence of rhythmicity in O_2-uptake in molluscs under laboratory conditions was further reported by Gompel (1937) and by Sandeen *et al.* (1953), and in the rate of water propulsion in the California mussel by Rao (1953). No prolonged interruptions of the ventilation were observed in the scallops and the oxygen uptake of the latter, accordingly, appears to be quite uniform (Figs. 5 and 6). Also, as already mentioned by Spärck (1936), scallops cannot be kept out of water for nearly as long a time as many other bivalves, without being damaged. When kept in moist air at room temperature they soon open their valves and gradually lose the typical reaction to the sudden cast of a shadow which consists of a vigorous closing movement. The oxygen consumption determined in a sea scallop which had been subjected to such a treatment for seven hours appeared to be low for the temperature of the test (74 ml. O_2/kg./hr., 22.7° C.), the critical oxygen tension high (test 3, Fig. 6). During this test the animal held its valves abnormally wide apart and its gills were arranged in a disorderly fashion.

(*b*) *Oxygen consumption at progressively decreasing concentrations of oxygen*

With not too rapid a fall in the oxygen content of the inspired water, the scallops appeared to be capable of maintaining a normal oxygen uptake down to a concentration of oxygen of about 1.0–0.5 ml. O_2/L. (Fig. 6). Similar findings have been reported for other species of Lamellibranchiata (Weiland, 1919; Nozawa, 1929; Galtsoff and Whipple, 1930; Ishida, 1935; Thamdrup, 1935; Whedon and Sommer, 1937; Hers, 1943). To what extent this faculty is based on an increase in the amount of water pumped or on an increase in the percentage oxygen utilization, has not been investigated. It was noticed that the animals, in water poor in oxygen, display little or no active motion, gradually lose the "shadow-cast reaction," and erect the pallial velum in a vertical fashion. It is possible that the vigorous stirring of the water in the respiration chamber has contributed to some extent to the ventilation of the animal's tissues with water and thus has lowered the critical oxygen tension below a level which would prevail if this ventilation depended solely on the currents generated by the animal itself.

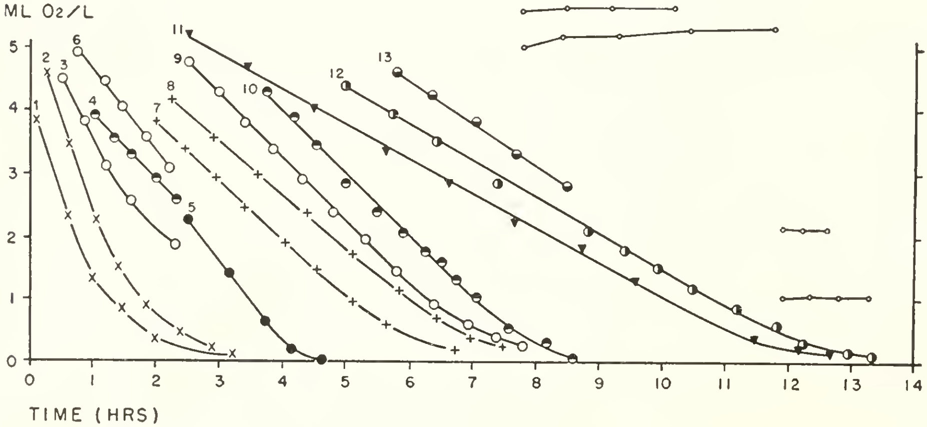

FIGURE 6. Rate of oxygen uptake of the sea scallop and of the bay scallop at progressively decreasing tensions of oxygen. Ordinate: O_2-content of water in respiratory chamber (ml. O_2/L.). Abscissa: time (hrs.). Numbering of tests as in Table I. For discussion of test no. 3, see text. Small circles: blank runs.

(*c*) *Oxygen utilization*

In the sea scallops the oxygen utilization was found to range from about ½ to 9%; in four out of the five bay scallops investigated the values represent a range of about 1 to 13% (Table II). Similarly low values have been found in siphonate Lamellibranchia and in several other filter feeding animals (van Dam, 1935b, 1938; Hazelhoff, 1938). Low values are also obtained for the oxygen utilization calculated from data on oxygen consumption and rate of water propulsion as given for a number of filter feeders, e.g., by Jørgensen (1952). It seems, then, that true filter feeders normally pump large amounts of water (*cf.* also Loosanoff and Nomejko, 1946; Owen, 1953), from which only a small fraction of the dissolved oxygen is being consumed. It would be of interest to determine the oxygen utilization or the rate of water propulsion in those species of bivalves which are not true filter feeders as, for example, those feeding on bottom deposits (cf. Yonge, 1953).

TABLE II

*Percentage oxygen utilization in the sea scallop**
O_2-content of inhaled water about 5 ml. O_2/L. Temperature about 17–22° C.

Specimen no. 1	Specimen no. 5	Specimen no. 6
2.9	7.5	1.9
2.2	6.4	2.7
0.7	5.2	0.8
1.6	7.6	3.6
1.6		2.3
1.6	Mean: 6.6%	2.3
4.0		2.9
8.8		3.4
1.8		1.9
4.0		3.8
2.2		3.8
2.2		3.8
0.4		
1.0		Mean: 2.8%
Mean: 2.5%		

* Except for one (Specimen no. 4, utilization 0.9%), the utilization values obtained in the bay scallop are shown in Figure 7.

In one of the bay scallops which ventilated much less vigorously than any of the other specimens investigated, high percentage oxygen utilization values (13–72%) were found. A similar, slow, often interrupted, ventilation entailing a high oxygen utilization was observed in some individuals of Anodonta (van Dam, 1938). In the specimens of Anodonta investigated by Koch and Hers (1943; personal communication) a high utilization prevailed throughout. Whether a slow rate of ventilation, as displayed by these specimens of Pecten and Anodonta, can be considered normal, *i.e.,* will provide the animal with enough oxygen and food to sustain normal, aerobic, respiration and growth, is not known.

In the stream of water escaping from the cloacal chamber of the bay scallops considerable gradients in the concentration of oxygen occur. These gradients are shown, in Figure 7, in the form of utilization values obtained simultaneously, but at different spots, in the exhalant water current of one and the same specimen. These values were connected by a line parallel to the ordinates. The steepest oxygen concentration gradients occurred in those cases where the utilization was high. In one of these cases (the group of three dots closest to the right hand ordinate) the utilization values were 33.4%, 37.4%, and 71.5%, respectively, representing oxygen concentrations of 66.6%, 62.6%, and 28.5%, of the oxygen content of the inhaled water. In this case, then, the concentration of oxygen at one spot was more than twice as high as at another spot. In the other, more vigorously ventilating specimens, in which the utilization was much lower, the differences in oxygen concentration did not exceed 10% of the O_2-content of the inhaled water.

Inhomogeneity in the distribution of oxygen in the exhalant water current as observed in the bay scallop by the present author and in *Anodonta* by H. J. Koch and M. J. Hers (personal communication), of course, excludes the possibility of determining the true average oxygen utilization with a sampling method. Only if all the water leaving the animal could be collected would the determination of such a value be possible. The oxygen utilization values, then, obtained in this and in previous investigations, are only approximations of this average value.

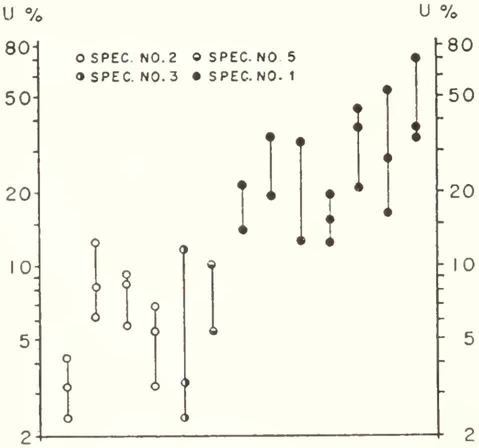

FIGURE 7. Gradients in the concentration of oxygen in the exhalant water current in the bay scallop, presented as the percentage oxygen depletion (U%) of samples drawn simultaneously at different spots in one and the same animal. U%-values from simultaneously drawn samples are connected by a line parallel to the ordinates. O_2-content inhaled water approx. 4.5–5 ml. O_2/L.; temperature about 20–24.5° C.

I wish to thank Dr. A. C. Redfield and Dr. P. F. Scholander for stimulating interest and valuable criticism, and Mr. J. A. Posgay for help in the procurement of scallops.

SUMMARY

1. The oxygen uptake and the percentage of oxygen withdrawn from the inhaled water, were determined in resting specimens of two species of lamellibranch mol-

luscs which have a capacity for rapid swimming locomotion, *viz.* in the sea scallop (*Pecten grandis* Sol.) and in the bay scallop (*Pecten irradians* Lam.).

2. In accordance with the absence of prolonged ventilation pauses common in several other species of Lamellibranchia, the oxygen uptake in the bay and sea scallop is quite uniform. In both species Q_{O_2} was found to be about 70 ml./kg./hr. at 20° C. This value is about half the Q_{O_2}-value found in swimming species from the mediterranean and falls well within the range of Q_{O_2}-values in boreal non-swimming types of lamellibranchs recorded in the literature.

3. The oxygen uptake was independent of the oxygen tension down to a concentration of oxygen of about 1-½ ml. O_2/L.

4. The percentage of oxygen withdrawn from the inhaled water, in most cases, was low, ranging, approximately, from ½ to 13%. It was demonstrated that in the stream of water escaping from the cloacal chamber of the bay scallop considerable gradients in the concentration of oxygen occur.

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STUDIES ON THE ACROSOME. II. ACROSOME REACTION IN STARFISH SPERMATOZOA [1]

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With the technical assistance of A. KITAHARA and T. KOHRI

After it had been found that sea urchin spermatozoa react to species egg-water by undergoing a change in the region of the acrosome simultaneously with the agglutination reaction, the possibility became evident that some such reaction might also be occurring in starfish spermatozoa. Fertilization in this form holds a peculiar interest, both because it was in the eggs of the starfish, *Asterias*, that Fol for the first time, in 1877, observed the entrance of a spermatozoan into an egg cell, and because his description of the process, which has never been superseded, contains certain points which are rather difficult to reconcile with usual concepts of the relationship between egg and spermatozoan.

Current ideas concerning the exact details of the process by which fertilization takes place in starfish eggs are in a somewhat surprising state of confusion, considering the number of embryologists who have observed and reported the phenomenon, and the extent to which this material has been used in various studies during the past seventy-five years.

Fol (1877) and Chambers (1923) observed spermatozoa being drawn toward a cone on the egg surface by a long, slender filament, which they believed to be an extension of the cone. Just (1929) repudiated this interpretation, and maintained that the much shorter filament which he observed originates in the sperm head. Hörstadius (1939) reported that a tubular "Empfängnishügel" grows out from the egg surface and takes possession of a spermatozoan at the outer edge of the jelly layer.

Similarly, although workers from the time of Lillie and Loeb have attempted to observe agglutination of starfish sperm in homologous egg-water, their results do not agree. Glaser (1914) and Woodward (1918) reported agglutination in *Asterias forbesii*, and Nomura (1926), in *Asterina pectinifera*, but Just (1930) was unable to confirm the *Asterias* results, and Loeb (1914) and recently Tyler (1941) have also reported lack of success with *Asterias ochraceus* and *Patiria miniata*, respectively. However, in 1944, Metz discovered that the addition of lobster serum caused an unequivocal agglutination reaction in the presence of homologous egg-water. Metz (1945) has further studied the phenomenon in four species of starfish, and found other substances which also act as adjuvants, particularly isotonic white of hen's egg.

The work reported in this paper was planned to test the effect of egg-water (plus an adjuvant) on the acrosome of the starfish spermatozoan.

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[1] This research was supported in part by the Ministry of Education Research Expenditure (Min-kan Kenkyu Hi).

MATERIAL AND METHODS

The starfish species used for the majority of the observations were *Asterina pectinifera* and *Asterias amurensis;* the main points were corroborated in *Astropecten scoparius.* Since the oocytes of *Asterina* and *Astropecten* in general do not begin the maturation divisions on suspension in sea water,[2] the egg-water in experiments with these forms was obtained from the immature eggs. In the case of *Asterias,* however, the egg-water was obtained from maturing eggs. No difference in effectiveness was found between the two solutions.

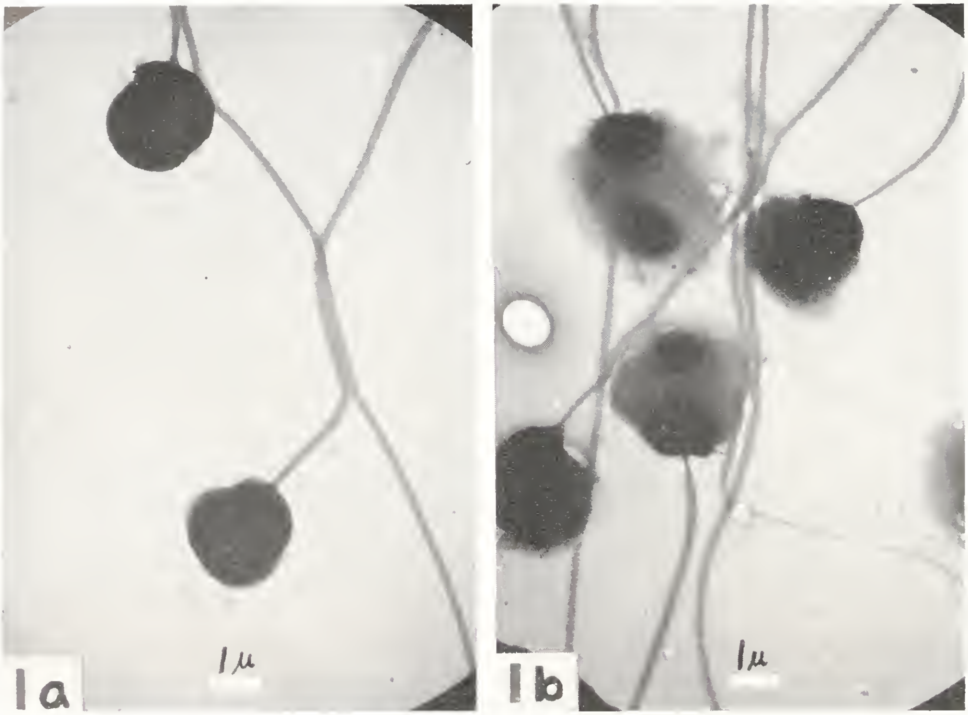

FIGURE 1. Electron micrographs of *Asterias* spermatozoa fixed in pure sea water, (a) with neutralized formalin; (b) with OsO_4 vapor.

In practice it was found that the jelly of *Asterina* oocytes swells considerably in Ca-low artificial sea water; after having stood for about 30 minutes, such suspensions were centrifuged sufficiently to remove at least part of the jelly, and 4% of 0.36 *M* $CaCl_2$ was added to bring the calcium content approximately to that of normal sea water. In the case of *Asterias,* sufficiently potent egg water was obtained by simply removing and filtering the supernatant fluid from a concentrated suspension of eggs which had stood for at least an hour.

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[2] However, there are always a few (less than 10%) of the oocytes in which the germinal vesicles do break down. These fertilize and develop normally.

Spermatozoa were obtained by extirpating the testes, which were kept "dry" in a covered glass container, and the sperm exuding from them was freshly suspended, immediately before use, in the adjuvant solution. Throughout the experiments, crystalline egg albumin, dissolved in sea water and filtered, was used as the adjuvant, in concentrations ranging between 0.1 and 1.0%.

Most of the observations were made on living sperm suspensions, with the oil immersion objective of a phase contrast microscope. For photographing and electron microscopic preparations, the suspensions were fixed with osmium vapor. Because of the presence of the dissolved albumin, however, it proved very difficult to obtain satisfactory preparations for direct electron microscopic observation. This difficulty was partially resolved by making replicas. The electron microscope was a Hitachi Standard, operating on 50 KV.

RESULTS

The head and middle piece of the starfish spermatozoan make up an approximately spherical structure, slightly flattened anteriorly (Fig. 1a). In the living spermatozoan a bluntly cone-shaped acrosome can be seen imbedded in the nuclear material so that the apex of the cone points backward, toward the middle piece (Fig. 2a). With phase contrast the acrosome material appears strongly refringent, in contrast to the material of the nucleus. The middle piece is relatively large, and is closely applied to the base of the head. While the combined length of the starfish sperm head and middle piece is only about half that of a moderately-sized sea urchin spermatozoan (*i.e., Hemicentrotus*), the tail is fully as long as any of the sea urchin sperm tails (50 μ). The head and middle piece are enclosed in a single membrane which is not apparent in normal specimens, but becomes visible

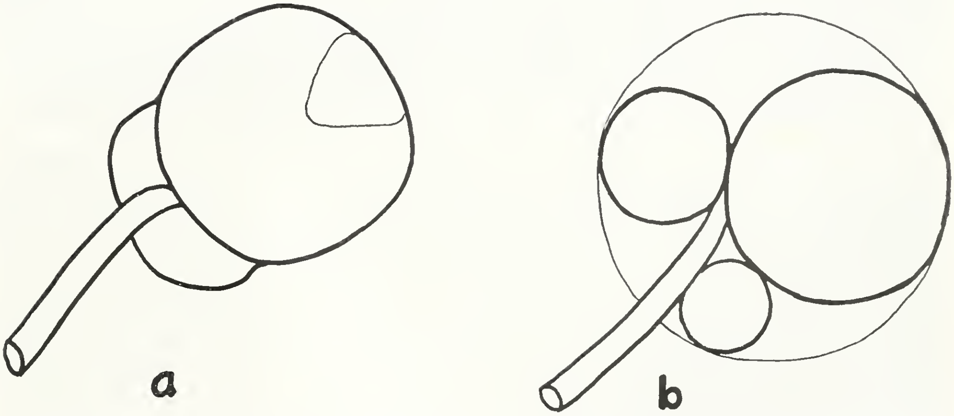
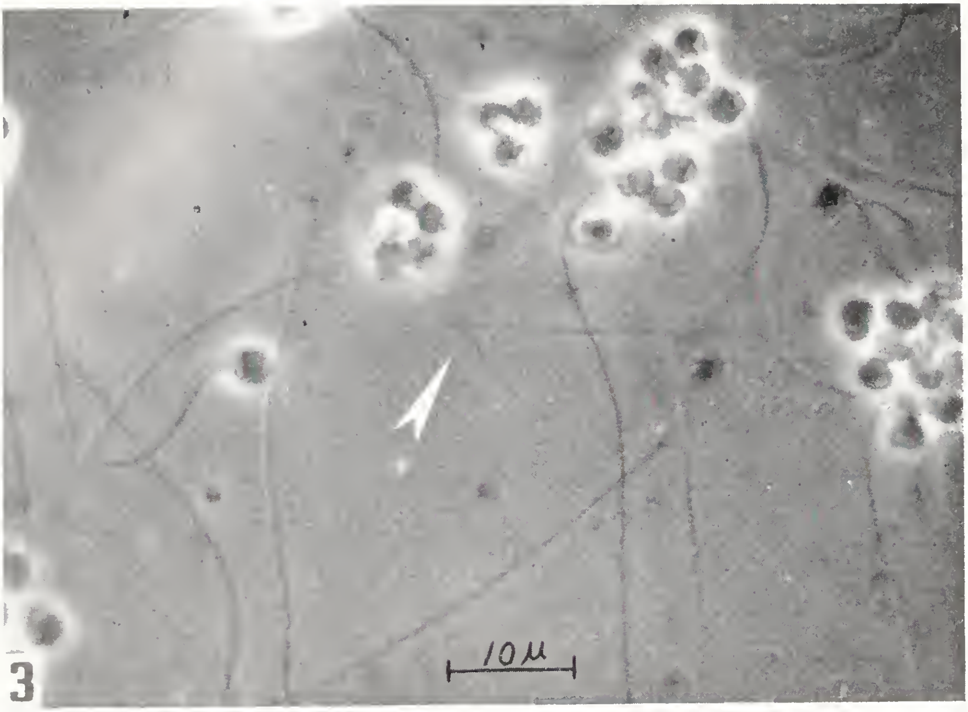


FIGURE 2. (a) Diagram of starfish spermatozoan in sea water. Middle piece is closely applied to posterior part of head; acrosome appears as a rounded cone imbedded in nuclear portion of head. Tail curves around middle piece and extends more or less directly backward. (b) Diagram of moribund starfish spermatozoan, showing nucleus, acrosome and middle piece rounded up separately within inflated membrane.



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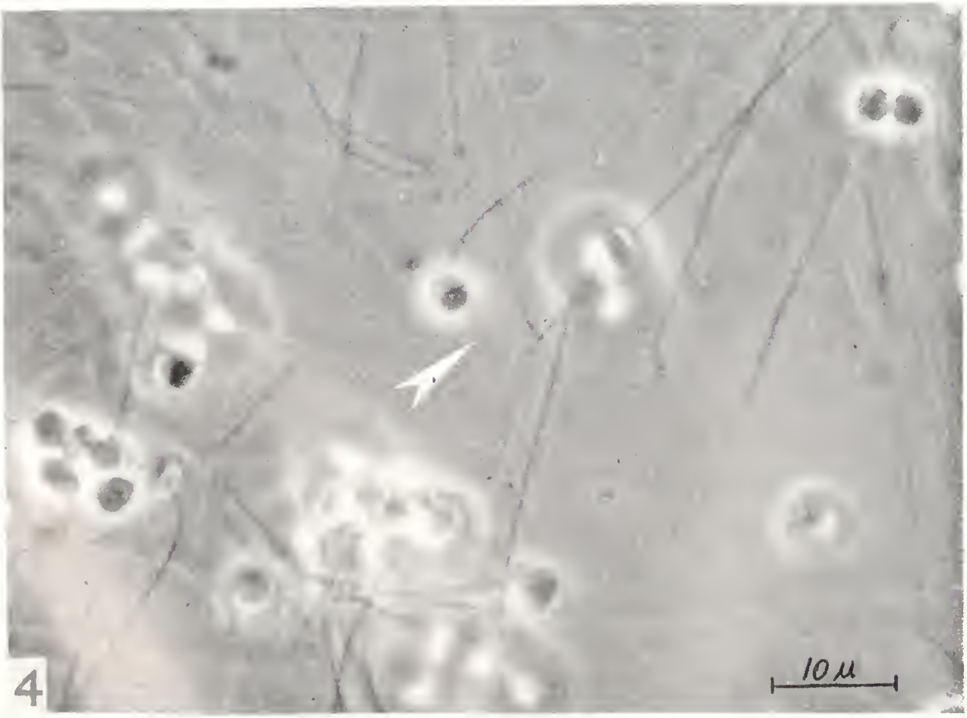

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Fig. 3–4.

in moribund sperm or following various types of treatment. In an aged suspension, moribund individuals are frequently seen in which the membrane has separated and become somewhat inflated, and within this spherical membrane the nucleus, middle piece and a refringent body, presumably the acrosome, are all to be seen as discrete spheres (Fig. 2b). The nucleus, middle piece and tail apparently retain their original connection with each other at one point, but the acrosome has moved out of place entirely and seems to be free within the membrane.

As Metz has reported, although starfish sperm are usually not appreciably activated either by suspension in sea water or by addition of homologous egg water, they become intensely active when they are suspended in egg albumin-sea water.

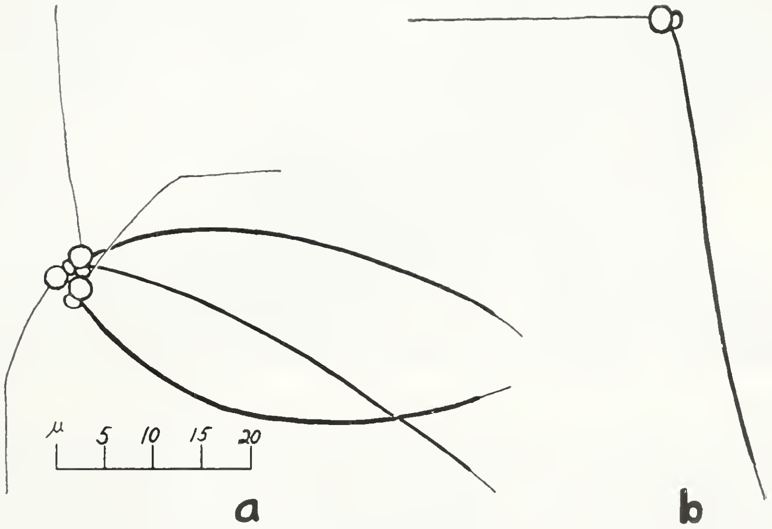

FIGURE 5. Camera lucida drawings of *Asterina* spermatozoa suspended in 0.5% albumin-sea water, after addition of homologous egg-water. (a) Small cluster of agglutinated sperm, showing the acrosome filament on each. A strong current of water was passed across this field from the left; two of the filaments were bent and all the tails swept toward the right side. (b) Single reacted spermatozoan held against cover glass by acrosome filament.

Such activated sperm, however, do not show any change in structure when they are examined either in the living state with phase contrast, or with the electron microscope.

If egg-water is then added to the suspension of spermatozoa in albumin-sea water, and a sample is observed under high magnification, many clumps including from a few to several hundred spermatozoa can be seen, the heads in contact with each

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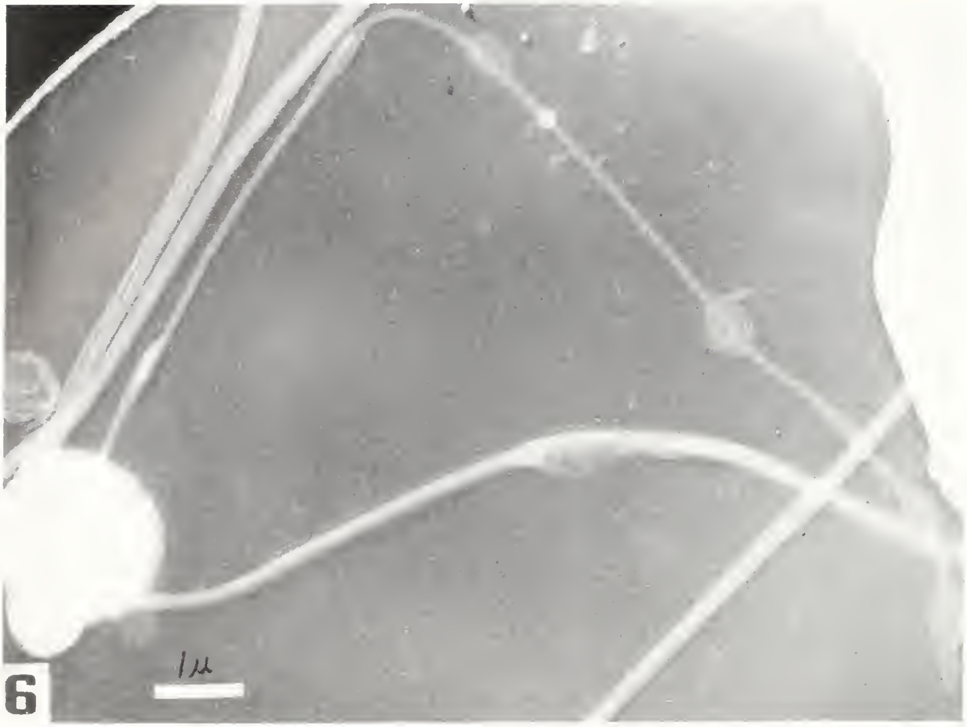
FIGURE 3. Phase contrast micrograph of *Asterina* spermatozoa suspended in 1% egg albumin-sea water, fixed with OsO_4 vapor after addition of homologous egg-water. Most of the spermatozoa are included in small head-to-head clusters; arrow indicates several acrosome filaments stuck to underside of coverglass.

FIGURE 4. Single *Asterina* spermatozoan which has reacted to egg-water focussed to show origin of filament in acrosome region of head.



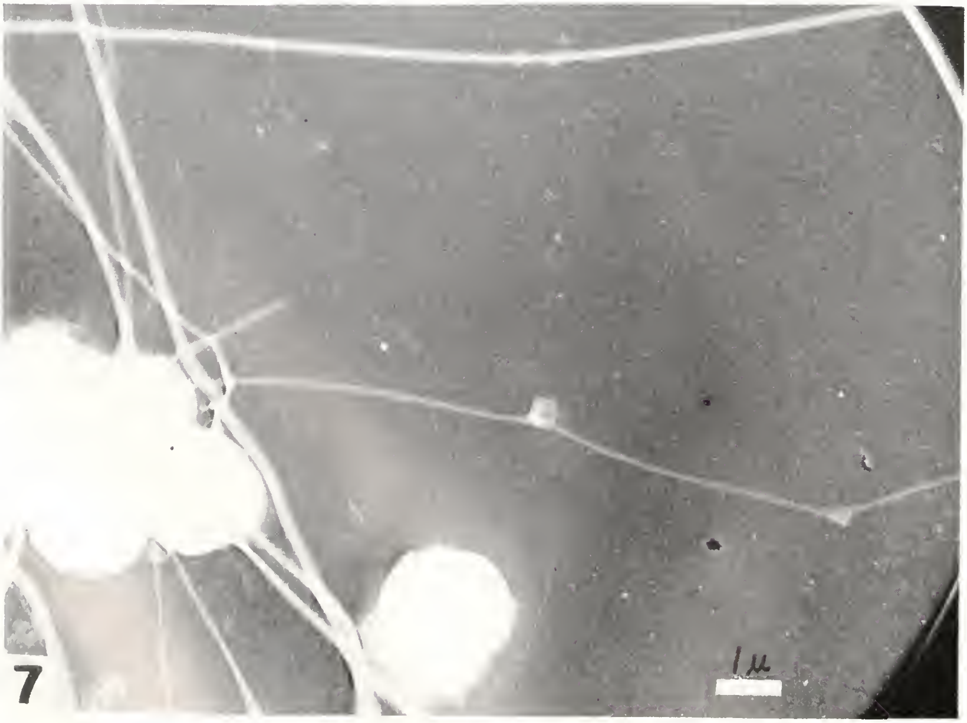


FIG. 6–7.

other, and the tails in vigorous movement (Fig. 3). This agglutination is irreversible.

Many other spermatozoa are in rapid solitary motion, and some can be found with their heads stuck against the glass surfaces, while their tails move freely. Close examination shows that these spermatozoa are held affixed to the glass by a long (ca. 25 μ), very slender filament which extends perpendicularly from the center of the acrosome surface (Figs. 3, 4 and 5a and b, 6). These filaments cannot be seen on swimming spermatozoa, but fixation of the suspension shows that this reaction of the acrosome has taken place in a large proportion of the cells. Especially in the case of the small agglutinated clusters, the filament can be seen on each spermatozoan (Figs. 5a, 7).

Measurements of camera lucida drawings of several of these filaments ranged between 22 and 28 μ. In the living state the filaments show considerable rigidity, bending only partially in the direction of a strong current of water passed over them, and returning to their original position when the current is stopped. They can also be seen to vibrate when hit by a passing spermatozoan, and are sometimes found broken, with part of the filament attached at an angle to the remainder. Some time after the addition of the egg-water, when the actively moving spermatozoa have become sufficiently stationary to permit observation, some of them can be seen to have short fragments of the filaments still projecting from the center of the acrosome surface. Apparently the rest has broken off, during their swimming

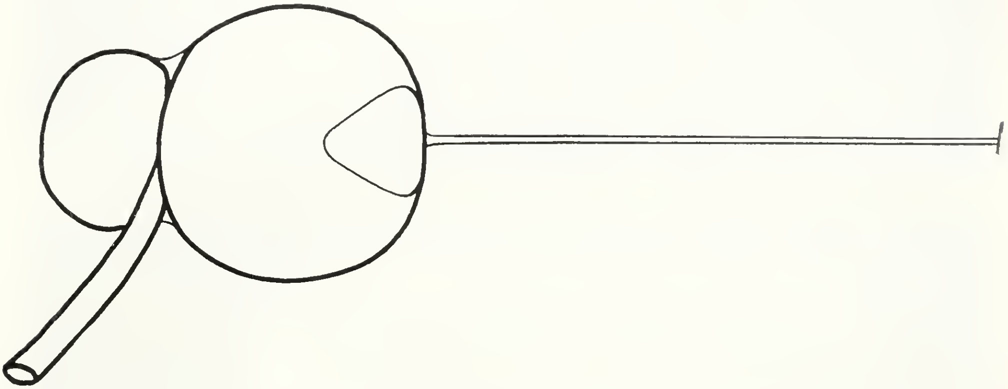


FIGURE 8. Diagram of starfish spermatozoan after acrosome reaction has taken place. Filament extends from center of acrosome surface; middle piece has become nearly spherical, and tail projects laterally, between head and middle piece. Only proximal portion of acrosome filament indicated.

FIGURE 6. Shadowed electron micrograph of *Asterina* spermatozoan; suspended in albumin-sea water and mixed with homologous egg-water on collodion membrane; fixed with OsO_4 vapor. Acrosome filament has been bent in handling, and vesicles formed at various points, probably as an effect of the fixative. Note that the membranous sheath of the filament seems to be closed at the tip, and that the central core gives evidence of spiral fibrillar structure.

FIGURE 7. *Asterina;* same treatment as above. Acrosome filaments projecting from agglutinated clump of spermatozoa.

about. Whether the filaments gradually disintegrate in sea water could not be determined definitely for this reason; it is certain that those which have been stuck to the glass from the beginning remain apparently unchanged for at least 30 minutes, but these are not fully exposed to a possible dissolving action of the sea water. No acrosome filaments could be seen on spermatozoa fixed with formalin, although they were well preserved after fixation with osmium vapor. This latter fixative, however, was quite ineffective with respect to the nuclear part of the sperm head, which became extremely flattened and diffuse in outline when the preparation was dried (Figs. 1b, 6, 7, 9, 10).

Besides the appearance of the acrosomal filament, there is a structural readjustment which takes place in response to the stimulus of egg-water and makes it possible to differentiate between spermatozoa which have reacted and those which have not. This consists in what appears to be a partial relaxation of the tight enveloping membrane, so that the middle piece rounds up and is less closely applied to the posterior part of the nucleus (Fig. 8; compare with Fig. 2a). Moreover, the tail, which in untreated spermatozoa curves around the middle piece before extending straight backward, now projects laterally from the posterior midpoint of the head (see also Figs. 5a and b, 6, 7, 8, 9, 10). This phenomenon was reported by Chambers (1930), who mentions an "impression that the head of the sperm is bent to one side" (p. 352), and further says (p. 352), "Occasionally a spermatozoan appears to be carried through the jelly with the base of its tail at right angles to the attachment of the insemination filament, while the rest of the tail is curved so as to trail behind." With phase contrast (dark contrast), the reacted acrosome region is no longer brightly refringent, appearing grayish and darker than the nuclear material.

Concerning the fine structure of the acrosome filament, not much can be said with certainty on the basis of the available electron micrographs. It appears to have a slightly greater diameter at the base than at the tip, although this difference cannot be detected in living specimens. There is definitely a central core, probably fibrous, surrounded by a sheath or membrane (Fig. 6). In fixed specimens, local blister-like swellings of this membrane are often found (Figs. 6, 7, 9). The end of the filament apparently has no special structure (Figs. 7, 9), although Figure 6 indicates that the tip is enclosed by the membrane. The knob-like condition of the filament tip in Figure 10 is apparently the result of fixation, and has no particular significance with respect to the acrosome filament itself, since the axial filaments at the ends of the sperm tails in the same preparation also reacted in the same way.[3] This at least suggests that the two slender filaments may have certain properties in common. They are further similar in that both appear to be rather sticky, since spermatozoa are often held firmly to the glass surfaces by the two filaments, while all the other parts—the head, middle piece and sheathed part of the tail—are movable against the glass.

In no case, in either *Asterina* or *Asterias*, has the acrosome reaction been found to take place in the great majority of the spermatozoa, as it does in sea urchins. Most of the sperm which fail to react have small, spherical heads, with the middle piece closely applied to the base of the head; while those which react to egg water

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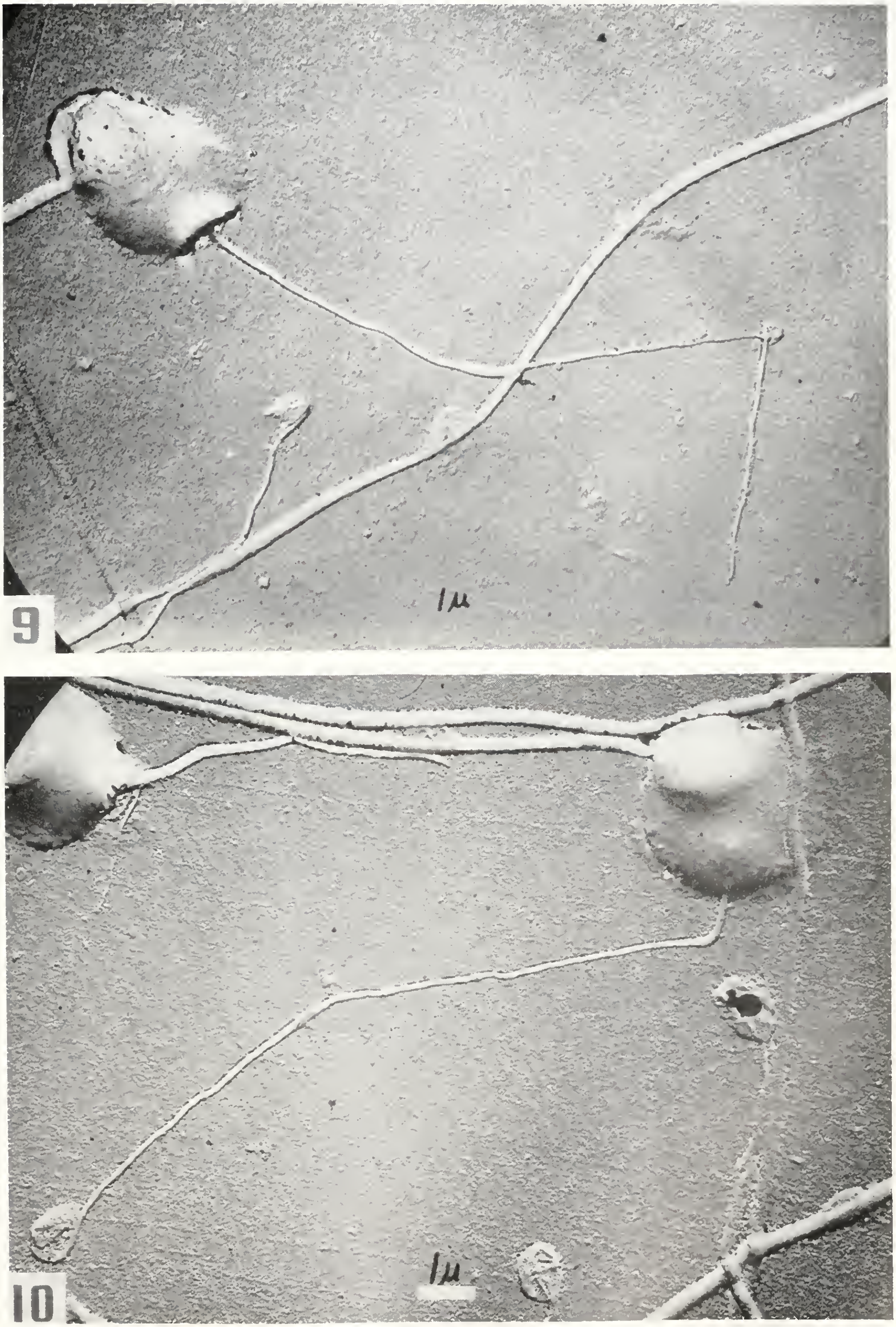
[3] These artifacts are no doubt identical with the vesicles formed at the tips of silver salmon sperm tails on fixation with osmium tetroxide and Zenker's fluid (Lowman, 1954).



FIGURES 9 AND 10. Replicas taken from *Asterias* spermatozoa suspended in 0.1% albumin-sea water, and fixed with OsO_4 vapor after addition of homologous egg-water.

are larger, the anterior part of the head, consisting of the acrosome surface, is flattened, and the middle piece is broad and prominent and its junction with the head is clearly differentiated. It seems possible that the spermatozoa of the smaller type may be non-functional.

Preliminary observations of fertilization in the three starfish species under consideration show that when insemination is carried out soon after the extrusion of the first polar body (see Delage, 1901), the eggs fertilize monospermically and develop normally, at least in the early stages. In all three species, within the minimum of 20–30 seconds which is required for insemination and adjustment of high dry (phase contrast) focus, the inseminating spermatozoan has become attached to the vitelline membrane by a very slender filament and is moving steadily through the dense jelly layer. About the time it reaches the vitelline membrane, this is being pushed away from the egg surface by a low fertilization cone. The spermatozoan traverses the intervening membrane and can be seen momentarily within the hyaline protoplasm of the cone, which becomes somewhat larger but is usually restrained during its developing phase by the very slowly rising fertilization membrane (see Dan, 1950, Fig. 7). By the time the sperm tail is nearly inside the egg, the membrane has lifted away from the degenerating cone, which rather quickly subsides in an irregular fashion and disappears completely within 6–10 minutes after insemination.

DISCUSSION

The fact that starfish spermatozoa can be shown to produce, in response to the stimulus of species egg-water, a long, slender filament which coincides in appearance with the filament supposed by various workers to proceed from the egg surface, necessitates a reconsideration of the evidence on which this supposition was based. Chambers (1930) cites Fol as having rejected the possibility that the filament originated in the spermatozoan because he was unable to detect any diminution in the size of the heads of spermatozoa attached to such filaments. For the following several reasons the decision seems unavoidable that Fol was mistaken in drawing such a conclusion:

1. the heads of starfish sperm are far from uniform in size, as observed in the living state, under phase contrast oil immersion;
2. estimates from observations of living sperm and electron micrographs indicate that the diameter of the living starfish sperm head is about 2 μ, which is too small, even with modern optical equipment, to permit accurate estimation of minor changes in volume;
3. calculations based on measurements of electron micrographs of osmium-vapor-fixed sperm heads and acrosome filaments indicate that loss of the volume of substance contained in a filament 25 μ in length and 0.13 μ in diameter would result in a reduction of the sperm head diameter of only about 2%;
4. the partial separation and rounding-up of the middle piece and main part of the sperm head at the time of the acrosome reaction produce sufficient change to obscure a much greater loss of substance than that necessary to produce the filament.

Fol's description of the fertilization process in echinoderms was so detailed and accurate that, as Chambers pointed out when he undertook a re-examination of the question nearly half a century later, "even recent textbooks comment upon the completeness of his observations" (1923, p. 821). Although Chambers referred to Fol's idea of the mechanism in starfish eggs, by which "the presence of spermatozoa in the immediate vicinity causes the egg to respond by forming on its surface a conical elevation which attracts the spermatozoan from a distance" as "rather extraordinary" (loc. cit.), he followed Fol in this interpretation, apparently in lieu of sufficient evidence to the contrary.

Chambers found that when starfish eggs are in the optimum condition for fertilization, the process takes place so rapidly that the filament is already extending between sperm head and cone tip by the time the earliest observation can be made. Relying on Fol's assumption of the "attraction cone" as the site of origin of the filament, Chambers records a number of observations in this and his later paper (1930) which convincingly serve to demonstrate its existence and show that it is the agency by which the spermatozoan is drawn through the jelly to the egg surface. However, the best evidence which he could muster, in the course of the two studies, for the origin of the filament in the attraction cone depends on the assumption that sperm entrance takes place in the same manner whether the eggs are in the optimum condition or either under- or over-mature.[4]

It is well known that in sea urchin eggs there are highly significant differences between the modes of sperm entrance, and more especially between the reactions of the eggs to insemination, under different conditions of maturity. In immature eggs there is apparently no block except spatial limitation to the entrance of any number of sperm; no fertilization membrane is raised; and the only apparent response of the egg cytoplasm to sperm entrance is the formation of conspicuous, semi-permanent structures on the surface which appear to be bundles of protoplasmic fibrils and bear little resemblance to the small, rounded, temporary cones of normal fertilization. Hobson (1927) found that in the oocytes of *Asterias rubens,* which had the germinal vesicles still intact and were "never observed to cleave or to complete their maturation" (p. 100), the reaction to sperm entrance was so similar to that reported by Seifritz (1926) in oocytes of *Echinarachnius parma* that he did not bother to repeat the description.

Since the optimal condition in sea urchin and sand dollar eggs lasts for a very long period, the various changes in the fertilization reaction associated with the passage of time may equally well be the result of a senescent loss of vitality, as of a condition of "over-maturity" such as that occurring in "post-optimal" starfish eggs, and there is probably little profit in attempting to compare the two sets of phenomena.

Just, on the other hand, attacked the interpretation of Fol and Chambers on the basis of his own observations of starfish eggs, which, he wrote, "indicate that maturated eggs lose the capacity for normal fertilization, and parallel with this loss runs the production of filaments." ". . . the production of filaments by *Asterias* ova . . . as a response to insemination is a phenomenon quite apart from the behavior in the normal fertilization reaction" (Just, 1929, p. 319). "*Fol used stale eggs, which accounts for the filament formation*" (op. cit., p. 322).

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[4] The terms "under-" and "over-mature" are used with reference to the "optimum condition for fertilization," which lasts only during the period between the extrusion of the first and second polar bodies.

The observations of other workers, then, do not support Chambers' primary assumption, that the fertilization reaction follows the same process regardless of the state of the egg with respect to the meiotic divisions. As a result, his conclusion based on this assumption, that since filaments have been observed to arise from the entrance cones in immature eggs and in old eggs, the filament in normal fertilization must necessarily originate in the same way, loses much of its validity.

With respect to eggs in the optimum condition for fertilization, Chambers says, "In fresh maturing eggs I have never been able to see the cone without also seeing the advancing sperm and the filament connecting the two. The formation of the filament is apparently too rapid" (1930, p. 354). This is the fact which remains when we cancel out of Chambers' observations the influence of Fol's dictum that the fertilization filament could not have arisen from the spermatozoan.

On the other hand, while Just insists that a strand which he observed connecting the sperm head with the fertilization cone "is a prolongation of the spermatozoon" (op. cit., p. 321), the fact that he based his observations partly on fixed material automatically raises a question as to their value. It seems obvious that such a process as the approach of a spermatozoan through the jelly to the egg surface cannot be preserved in successive stages by any of the ordinarily used methods of fixation, since they either cause the jelly layer to shrink or dissolve it completely away. In the best case the exact relation of the sperm to the egg surface could only be captured by fixation after the spermatozoan had become firmly attached to the egg cytoplasm.

At least the first part of Just's observation seems to have been made on living material: "The intensely active spermatozoa rush toward the jelly hull; of these, one rapidly moving through it reaches the egg within 5 seconds after insemination" (loc. cit.). In the experience of Chambers, as well as of the writer, it has been found that at least twenty or thirty seconds are required to bring the freshly inseminated eggs into high power focus and discover one with a fertilizing spermatozoan attached exactly in the largest optical section. The sperm is by this time already connected with the egg surface by the filament in question, and according to Chambers' figures (1923, Fig. 2, a-c; 1930, Fig. 2, A-H), about one minute more is required for the sperm to pass through the jelly layer and reach the surface of the vitelline membrane. Preliminary observations by the writer on *Asterias amurensis* showed the sperm head reaching the membrane surface about two minutes after insemination (at 17° C.). In *Asterina pectinifera*, the sperm passed through the narrow jelly layer within 50–60 seconds after insemination (23° C.). *Astropecten scoparius* has a somewhat wider jelly layer, which the sperm crossed in about seventy seconds (at 25° C.).

A more detailed study of the fertilization reaction in these three species will be presented in a later paper; these preliminary data are cited as the basis of the writer's inability to accept Just's figure of five seconds as the time required for a spermatozoan to traverse the jelly layer of the *Asterias* egg.

Just further describes the process as he saw it in both living and fixed preparations: "As the cone grows the spermatozoon is pushed off from the egg, a delicate strand connecting the tip with the apex of the cone. This strand never attains the length given by Fol, and of course could never therefore be equal to the greater length figured by Chambers" (1929, p. 321). Just does not explain how or why such a structure is produced, beyond stating categorically, "*this strand is a prolongation of the spermatozoon, the tip of which is fixed within the cone*" (Just's italics; op. cit., p. 321). In the conclusion of his paper, however, he draws a comparison which probably reveals the source of his confidence in making the above statement: "This cone in *Asterias* egg closely resembles that in *Nereis* egg," except for the time factor. "Moreover, the strand between the sperm head and the cone in both cases has the same origin, namely, from the spermatozoon itself" (op. cit., p. 324).

It seems doubtful whether this analogy provides a sufficiently firm basis for a flat rejection of Chambers' observations as well as his interpretation.

Since Hörstadius, in describing fertilization in *Astropecten aranciacus*, does not mention filaments of the order of size of those under consideration, a discussion of his interpretation of the process is not strictly appropriate at this time. However, since the writer has clearly observed the formation of acrosome filaments in response to egg-water in the spermatozoa of *Astropecten scoparius*, and also in the fertilization process, it appears likely that in *A. aranciacus*, also, filaments from the sperm acrosome give the stimulus for the elevation of the cylindrical cones observed by Hörstadius. That author himself suggests that the formation of very many large cones is a characteristic response of immature eggs to insemination.

To summarize this discussion concerning the origin of the "fertilization filament"—it appears that, among later workers, only Chambers actually observed the filaments first discovered by Fol. Just's categorical statement quoted above with respect to the length of the filament, as well as the sequence of steps in its formation and their timing, all indicate that he failed completely to see the structure described in the earlier papers. Moreover, since Chambers, in spite of his accurate observation, was unable to establish conclusively the origin of the filament in the "attraction cone," we find the whole weight of this explanation of starfish fertilization resting upon Fol's assumption. This, in turn, depends upon his belief that he should have been able to observe a difference in the size of sperm heads before and after the extrusion of the filament.

If the criticisms of this assumption listed above be allowed, it follows that no incontrovertible evidence has been presented proving that the filament, observed by Fol, Chambers and the writer connecting the sperm head with the egg surface, arises from the egg. There is, moreover, no other case in animal fertilization, so far as the writer is aware, in which an egg has been shown to reach out and capture a spermatozoan.

On the positive side, evidence has been found (unpublished data) to show that the acrosomes of sea urchin and sand dollar spermatozoa undergo a reaction, in response to the stimulus of species egg-water, which can most readily be interpreted as providing a method of penetrating the first serious obstruction[5] which the spermatozoa encounter in their progress toward the egg pronucleus—the vitelline membrane. Since the tip of the sperm head is already in contact with the membrane, the liberation of a lytic substance, together with the reaction of the egg cytoplasm would presumably be sufficient to effect penetration. In the case of the starfish egg, on the other hand, an impenetrable jelly layer intervenes between the spermatozoan and the living egg surface. This necessitates the extrusion of a projection of the acrosome, long enough to cross the jelly layer, and sufficiently rigid to penetrate the vitelline membrane and establish contact with the reactive cytoplasm. It is believed that these conditions are fulfilled by the filament which is extruded from the starfish acrosome in response to the presence of species egg-water.

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[5] In these species, the spermatozoa swim through the jelly layer in a radial direction with at most only a slight reduction in speed.

At present there is no evidence as to how the filament is drawn into the egg cytoplasm. That such a process is entirely possible is shown by the fact that in the sea urchin, *Mespilia globulus*, the entrance of the sperm tail may take place very slowly (Dan, 1950), so that the sperm aster appears and even syngamy is complete before the tail is more than halfway into the egg. In this case it is impossible to imagine that the motionless tail is proceeding under its own motive power; nevertheless, its whole length is taken into the cytoplasm within about 15 minutes.

Tyler (1948) has suggested an explanation of the starfish filament in terms of a fertilizin-antifertilizin interaction, in which active antifertilizin groups on the sperm would combine with active fertilizin groups on the micelles of the jelly, resulting in precipitation, or contraction, of the micelles. Since these are assumed to be anchored to the surface of the egg, their contraction would draw the spermatozoan a slight distance toward the egg and into contact with new micelles, where the reaction would be repeated, until the sperm head finally reached the egg surface.

This is an ingenious attempt to fit the starfish fertilization process into the general echinoderm pattern, and represents a decided advance beyond the appeal to a mysterious force directing an attraction cone filament to a waiting spermatozoan. Observation of the actual fertilization process shows, however, that the filament has already attained its full diameter while the sperm head is still nearly the whole width of the jelly layer away from the egg surface, and no change in this diameter can be detected as the sperm head approaches the egg. Moreover, it seems questionable whether a filament of microscopically observable dimensions could be produced by the mechanism suggested, even given a radial arrangement of the submicroscopical jelly micelles. Finally, it must obviously be quite hopeless to rely on this scheme for an explanation of the filaments found on egg-water-treated spermatozoa.

The abrupt extension of a long, slender filament is a phenomenon already familiar in the discharge of trichocysts by ciliates and nematocysts by the coelenterates. Since the acrosome of the starfish spermatozoan measures only a fraction of a micron, it seems hardly possible that any very complex mechanism could be contained within it. This is one consideration in favor of a trichocyst-like discharge mechanism. However, the problem is within the range of direct experimental attack and further discussion will be reserved until evidence is available.

The writer gratefully acknowledges the unfailing cooperation of the staff of the Misaki Marine Biological Station; the hospitality of the Akkeshi Marine Biological Station of Hokkaido University; and the courtesy of the Tokyo Institute of Technology in placing its electron microscope at her disposal.

SUMMARY

1. When the spermatozoa of three starfish species, *Asterina pectinifera, Asterias amurensis* and *Astropecten scoparius,* are suspended in a dilute (0.1–1.0%) solution of egg albumin in sea water and mixed with homologous egg-water, three effects can be noted:

a. many of the spermatozoa are agglutinated by their heads, forming permanent clusters;
b. from the center of the acrosome of each agglutinated spermatozoan there has been extended a long (ca. 25 μ), very slender, straight filament which possesses considerable rigidity;
c. in all the spermatozoa which have so reacted, there is a rearrangement of the principal parts, so that the middle piece is less tightly apposed to the head and the tail appears to be inserted laterally, between the head and middle piece.

2. A critical examination of the widely accepted explanation of starfish fertilization—that the effective spermatozoan is drawn through the jelly layer to the egg surface by a filament originating in an "attraction cone"—shows that this depends mainly upon an assumption made by Fol, which various considerations show to be of doubtful validity. In the light of the fact that starfish spermatozoa produce a similar filament from their acrosomes, on contact with dissolved jelly substance, it is proposed that this sperm acrosome reaction is the source of the filament which, extending through the jelly, stimulates the egg cortex. Following this stimulation, the egg cytoplasm draws in the filament with the attached spermatozoan and simultaneously forms a fertilization cone beneath the vitelline membrane, which separates as the fertilization membrane. This sequence of events is the same as that constituting the fertilization reaction in other echinoderms.

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COMPARATIVE STUDY OF THE GILL AREA OF MARINE FISHES

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Among fishes there are obvious species differences in number of gill arches, as well as in number and length of gill filaments. Less obvious are species differences in number and size of the respiratory units, the gill lamellae. This suggests that there is also variation in gill surface area per unit of size of fish. Since fishes occupy a variety of ecological niches it is of interest to know if the gill area may in any way be correlated with activity and habitat.

Although there have been many studies on the anatomy of fish gills, only a few investigators have concerned themselves with gill area. Riess (1881) was among the first to attempt accurate measurements of gill surface. He found that pike had 125 sq. mm. of respiratory surface per gram of body weight. Putter (1909) determined gill surface area of a few fishes, particularly *Scorpaena*, and felt that the respiratory surface was proportional to body surface but not to body weight as maintained by Riess. The most extensive study on a single species was made by Price (1931), who followed the changes in gill area throughout the development of small-mouthed bass. The only study of a comparative nature appears to be that of Schöttle (1931), who found a reduction of gill surface in terrestrial gobiiform fishes compared to strictly aquatic species.

The studies reported here were carried out exclusively on marine teleost fishes representing twenty-three families and thirty-one species. The work was done in part at the Marine Biological Laboratory, Woods Hole, Mass., and in part at the Duke University Marine Laboratory, Beaufort, N. C. Partial support came from the Duke University Research Council.

For permission to participate in a cruise of the *Albatross* in North Carolina waters when several dolphin were obtained, and for use of laboratory space during part of one summer, grateful acknowledgment is made to the Institute of Fisheries Research of the University of North Carolina and to the U. S. Fish and Wildlife Station at Beaufort, respectively.

METHODS

Each gill filament is made up of numerous respiratory units, the lamellae or platelets. To obtain the respiratory area of a fish it is necessary to know the total area of the lamellae. It is, of course, impractical to measure each of the thousands of lamellae present; consequently a sampling method must be employed.

After weighing the fish and making outline drawings for later determination of body surface area, the gill arches of one side were dissected out, carefully separated, and placed in dishes of sea water, one arch to each dish. The number of filaments on each side of each arch was counted under a dissecting microscope. The average length of the filaments was determined by measuring every tenth filament with vernier calipers. From these measurements the average length of the filaments was established. By placing filaments of approximately average length under the low power objective of a compound microscope and using either a stage or an ocular micrometer, the number of lamellae per millimeter of filament was determined. What appeared to be lamellae of average size were separated from filaments of average size and placed on slides. Camera lucida drawings were then made of several lamellae. Usually this could be done using the lower power objective of a compound microscope. Planimeter readings were made of the camera lucida drawings and an average taken.

Knowing the number of gill filaments, the average length of the filaments, and the number of lamellae per millimeter of filament, the total number of gill platelets was readily determined. From this and the known magnification and area of the camera lucida drawings, the total area of gill surface could be computed.

In those species with extremely delicate filaments, the task of obtaining undamaged platelets was facilitated by hardening the filaments for a few minutes in formaldehyde or Bouin's fluid. The central cartilaginous support of the filament that runs through each lamella was not included in the computation of area. Since the lamellae are functional on both sides the determined area was doubled.

Admittedly, with so many manipulations, there is a large possibility of error in determining gill area. However, all specimens were treated in the same manner so that the results obtained would be comparable.

Body surface area of most species was found by drawing around the fish, allowing for body thickness and omitting dorsal, anal and paired fins, and determining the area of the outline with a planimeter. This method is quite adequate for depressed and compressed fishes, such as goosefish and butterfish. It can be used with more difficulty with odd-shaped fishes like sea robins, puffers, and toadfish. The body surface areas of these latter species were also determined by the more laborious method of covering the body with pieces of paper and making planimeter readings on each piece. The use of a general formula for determining body surface area was discarded because the different species differed in form and weight and a correction factor for each species would first have to be obtained. This has been discussed in a previous paper (Gray, 1953).

Results and Discussion

In Table I, which includes 31 species representing 23 families of marine teleosts, it can be clearly seen that species differ widely in number of respiratory lamellae and in respiratory area. This is true whether comparison is based on unit of body weight or on unit of body surface. In this table species are arranged in descending order of gill area per square centimeter of body surface. The contrast in respiratory area of those species near the top when compared with those near the bottom is great. One familiar with common marine fishes of the Atlantic coast will see a general correlation between respiratory area and species activity.

For convenience of discussion, and with no sharp demarcation between them, the fishes may be divided into three groups.

I. Active, schooling, migrating, fast swimming, streamlined fishes that, at least in the adult stage, seldom frequent the smaller estuaries. To this group clearly belong the mackerels, menhaden, dolphin, bluefish. At least during the summer months these species are in constant motion as they feed on plankton or follow schools of smaller fish.

II. Fishes of moderate activity, estuarine species limited in their daily travels. These include such species as scup, sheepshead, sea bass and others that hang around jetties, wrecks, piers, etc., feeding on crustaceans, molluscs, and sessile animals. On the lower fringes of this group may be included tautog, sea robins and puffers that spend part of their time resting on the bottom.

III. Relatively sluggish species more or less adapted for benthic life. Toadfish, goosefish, and flounders are good examples.

Table I

Gill area of marine fishes

Species of fish	No. of determinations	Average weight (gms.)	Lamellae per mm. of filament	Gill Area (sq. mm.)					
				Per gram of body weight			Per sq. cm. of body surface		
				max.	min.	ave.	max.	min.	ave.
False albacore, *Gymnosarda alleterata*	1	5216	33	1939	1939	1939	4854	4854	4854
Menhaden, *Brevoortia tyrannus*	12	613	29	2547	1241	1773	2704	1300	1828
Dolphin, *Coryphaena hippurus*	4	4015	26	965	618	710	1334	971	1169
Bonito, *Sarda sarda*	2	2192	31	631	558	595	1237	1073	1155
Bluefish, *Pomatomus saltatrix*	1	1035	29	652	652	652	841	841	841
Common mackerel, *Scomber scombrus*	15	182	31	1532	802	1158	1103	543	838
Spanish mackerel, *Scomberomorus maculatus*	2	478	29	770	768	769	731	724	728
Jumping mullet, *Mugil cephalus*	9	166	27	1105	760	954	845	538	654
Hard tail, *Caranx crysos*	3	129	39	1048	894	982	610	567	594
Striped bass, *Roccus lineatus*	4	3059	19	426	148	302	818	415	592
Sheepshead, *Archosargus probatocephalus*	6	2366	19	467	212	328	553	333	488
Bur fish, *Chilomycterus schoepfi*	2	316	13	449	425	437	492	482	487
Scup, *Stenotomus chrysops*	7	395	26	623	391	506	612	352	478
Tautog, *Tautoga onitus*	6	580	18	519	293	392	531	317	435
Red-winged sea robin, *Prionotus strigatus*	11	460	20	768	318	483	642	191	424
Butterfish, *Poronotus triacanthus*	9	199	31	817	386	598	572	252	411
Sea trout, *Cynoscion regalis*	6	807	27	593	221	373	671	253	410
Rudderfish, *Palinurichthyes perciformis*	6	199	24	658	377	506	550	268	388
Remora, *Echeneis naucrates*	2	393	22	783	314	549	—	—	—
Puffer, *Spheroides maculatus*	13	250	16	956	324	470	585	232	372
Sea bass, *Centropristis striatus*	4	244	21	653	439	458	480	233	361
Goosefish, *Lophius piscatorius*	3	6392	11	267	116	196	360	245	299
Harvest fish, *Peprilus alepidatus*	2	71	26	526	483	505	257	230	244
Conger eel, *Leptocephalus conger*	2	2560	19	136	134	135	224	202	213
Brown sea robin, *Prionotus carolinus*	2	213	20	394	326	360	225	197	211
Cutlass fish, *Trichiurus lepturus*	3	116	31	627	361	536	244	138	198
Common eel, *Anguilla rostrata*	4	428	19	398	183	302	236	125	193
Fluke, *Paralichthys dentatus*	5	766	19	328	206	242	225	134	176
Flounder, *Pseudopleuronectes americanus*	2	734	18	218	183	200	134	115	125
Toadfish, *Opsanus tau*	58	233	11	362	94	197	226	69	125
Sand flounder, *Lophopsetta maculata*	1	411	20	188	188	188	90	90	90

The numbers of lamellae per mm. of gill filament are also included in Table I. Here again it is found that, in general, the most active fishes have smaller lamellae placed closer together than do the more sluggish species. Usually a large number of small lamellae means more surface than a small number of large lamellae. But of course other factors such as the length of the filaments, the number of filaments and the number of gills are also important in determining the amount of gill surface.

Fishes with large lamellae spaced far apart often live longer out of water than those with closely packed fine lamellae. A toadfish will live for hours on the laboratory floor; a butterfish dies in a matter of minutes. Delicate closely spaced lamellae adhere together when removed from an aquatic medium and the functional surface is thus greatly reduced. It is the sluggish fishes with low metabolism that have the widely spaced lamellae.

In gobies, Schöttle (1931) has shown that those capable of remaining out of water have gill lamellae so arranged as not to collapse when the fish is on land.

Criteria for estimating degree of activity are unfortunately not based on oxygen consumption. It is desirable but difficult to obtain comparative metabolism data on marine fishes. From necessity most data are obtained from sluggish and moderately active species. Active species are hard to keep in captivity, or even to get to the laboratory, without becoming partially asphyxiated. Some, and this has been observed particularly in the family Scombridae, apparently die quickly from nervous exhaustion. This is especially true of schooling fishes when separated from their companions. However, the fact that it is difficult to maintain these fishes in the laboratory is at least a qualitative indication of relative activity even though not a quantitative one. It is these species that have the greatest relative gill surface.

Table II

Order of rank of marine fishes based on number of lamellae and on gill area

Gill lamellae per mm. of filament		Gill area per gram of body weight (sq. mm.)		Gill area per cm. of body surface (sq. mm.)	
1. Mackerel	31	1. Menhaden	1773	1. Menhaden	1828
2. Butterfish	31	2. Mackerel	1158	2. Dolphin	1169
3. Menhaden	29	3. Mullet	954	3. Mackerel	838
4. Mullet	27	4. Dolphin	710	4. Mullet	654
5. Sea trout	27	5. Butterfish	598	5. Striped bass	592
6. Dolphin	26	6. Scup	506	6. Sheepshead	488
7. Scup	26	7. Rudderfish	506	7. Scup	478
8. Rudderfish	24	8. Sea robin	483	8. Tautog	435
9. Sea bass	21	9. Puffer	470	9. Butterfish	429
10. Sea robin	20	10. Sea bass	458	10. Sea robin	424
11. Striped bass	19	11. Tautog	392	11. Sea trout	410
12. Sheepshead	19	12. Sea trout	373	12. Rudderfish	388
13. Eel	19	13. Sheepshead	328	13. Puffer	372
14. Fluke	19	14. Striped bass	302	14. Sea bass	361
15. Tautog	18	15. Eel	302	15. Eel	193
16. Puffer	16	16. Fluke	242	16. Fluke	176
17. Toadfish	11	17. Toadfish	197	17. Toadfish	125

Researches of others on some of the same species lend support to the theory that the active fishes have higher metabolism than bottom dwellers. Hall and Gray (1929) in their hemoglobin studies showed that the mackerel with 43 and the menhaden with 41 were relatively high in mgs. of iron per 100 cc. of blood, in contrast to the goosefish with 14.7, toadfish, 13.5, and sand flounder, 11.5. Scup (25.3), sea robin (23.7), and puffer (21.5) were intermediate. Also, Hall and McCutcheon (1938) have pointed out a correlation of hemoglobin function with activity and habitat in marine fishes. Mackerel and toadfish represented the extremes of the fishes they studied, with scup occupying an intermediate position.

Similarly, Gray and Hall (1930) showed that active fishes had greater amounts of blood sugar per 100 cc. of blood than did less active species (menhaden 75 mgs., mackerel 64, scup 53, sea robin 37, puffer 23, toadfish 15, and goosefish 6).

Vernberg and Gray (1953) found that a correlation exists between oxygen consumption of excised brain tissue and activity in fishes. The O_2 consumption of menhaden brain was found to be nearly twice that of toadfish brain, with moderately active fishes occupying an intermediate position.

Thus it seems that there are many correlations with activity and it is not surprising that there should also be a correlation between gill area and activity.

A just criticism of Table I could be that in many cases only one or two determinations were made and with the possibility of such a large potential experimental error there might arise a question of accuracy. However, if we consider only the seventeen species for which four or more determinations were made, there still remains a definite correlation with activity. This is shown in Table II where rank in order of number of lamellae per mm. of filament, gill area per gram of body weight and gill area per square centimeter of body surface are given. The order shifts a little in the three columns but the principle still holds: active fishes in general have more lamellae per mm. of filament, and greater respiratory area per unit of weight or per unit of body surface.

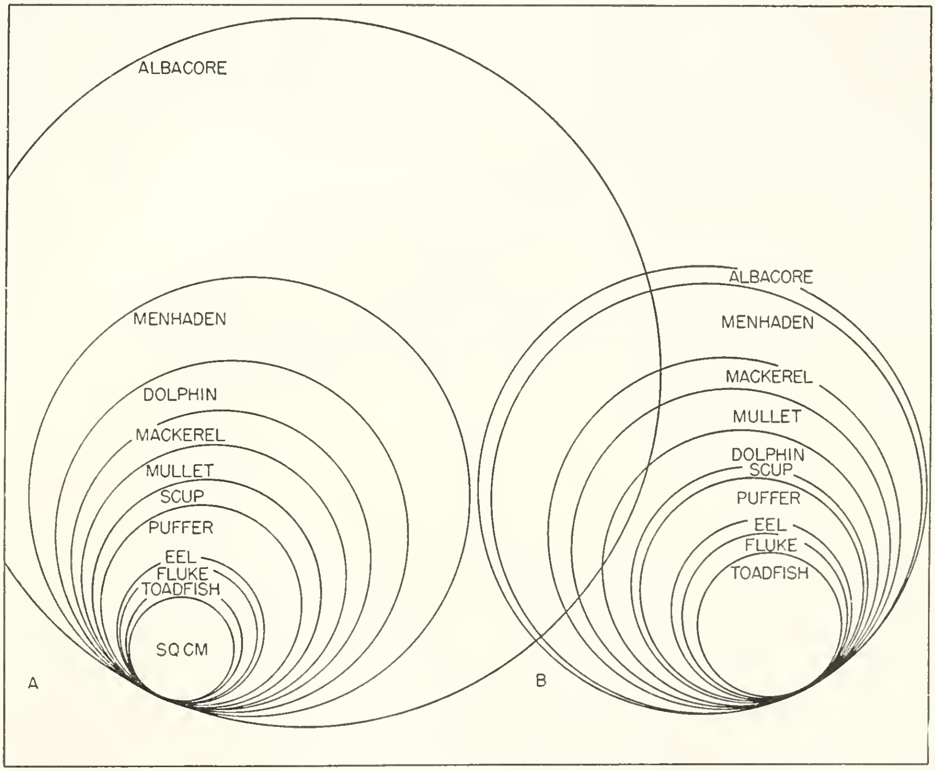

FIGURE 1. Relative gill areas of marine fishes. A. Per sq. cm. of body surface area. B. Per gram of body weight.

A more serious criticism is that the fish studied are not of the same size. This is especially significant when using body surface area as a basis for comparison, for a large fish has a relatively smaller body surface area in proportion to body weight than does a smaller fish of the same species. At first glance, the respiratory area of the false albacore per square centimeter of body surface may seem out of line. Unfortunately only one determination was made so that there can be only speculation as to accuracy. However, the surface area of this fish was in line with that of other scombrids (Gray, 1953), and gill area determinations were rechecked. This is a muscular, fast swimming fish with relatively small body surface area and it is to be expected that the ratio of respiratory area to body surface area would be high. Attempts to obtain gill areas of larger ocean fishes such as tuna were in vain. If one may be permitted to speculate, the prediction is that when obtained, the respiratory area of a large tuna in relation to body weight will be found to be near that of the albacore, but will exceed that of the albacore by many times when compared to body surface area.

TABLE III

Respiratory area of fishes of the same weight

Fish	Weight (gms.)	Total gill area (sq. mm.)
Menhaden	540	821,079
Sheepshead	544	254,237
Tautog	547	198,201
Toadfish	560	72,871
Menhaden	620	858,322
Toadfish	620	86,867

Figure 1 shows graphically the relative gill area of several species of teleost fish, representing different degrees of activity, compared both on the basis of unit of body surface (A) and on unit of body weight (B). The false albacore illustrates clearly the difficulty in comparing fishes of vastly different sizes on the basis of body surface. This streamlined fish was not only much larger than the others but also had an extremely large gill area. It seems to the author that when comparing gill areas of fishes of different species and differing greatly in size the comparison is more satisfactorily made when based on unit of weight than on unit of body surface area. Certainly the weight can be obtained much more easily and accurately than can body surface area.

Table III shows that when fishes of approximately the same weight are compared there is a definite species difference in respiratory area. It is seen here that the respiratory area of the active menhaden is roughly ten times that of the sluggish toadfish of the same weight, and that the moderately active sheepshead and tautog occupy an intermediate position.

SUMMARY

1. The gill areas of 31 species of marine teleost fishes have been compared.
2. Active, fast swimming, schooling, streamlined fishes (such as menhaden, mackerel, bluefish) have relatively much greater gill areas than do sluggish, benthic

species (toadfish, goosefish, flounders). Fishes of moderate activity (scup, sheepshead, sea bass, sea robin, puffer) are also intermediate in gill area.

3. Species differences in gill area exist whether comparison is based on unit of body surface area or on unit of body weight.

4. In general benthic fishes have fewer gill lamellae spaced farther apart than do fast swimmers.

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NITROGEN METABOLISM OF THE SLIME MOLD DICTYOSTELIUM DISCOIDEUM DURING GROWTH AND MORPHOGENESIS[1]

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Biologists concerned with the biochemistry of developing organisms would profit from analyzing a system in which the processes of growth occur independently from those of morphogenesis. The desirability of such a system has been emphasized by Needham (1942). The slime mold, *Dictyostelium discoideum* Raper, an organism exhibiting this phenomenon in nature, has been described by numerous authors, among them being Raper (1941) and Bonner (1944, 1947). Hirschberg and Rusch (1950, 1951) have indicated the suitability of the slime mold for studies of the biochemistry of development.

In view of current findings, however, there would seem to be no further justification for assuming the morphogenetic phase of development of *D. discoideum* as being devoid of growth processes (Wilson, 1952, 1953). In consequence it may be necessary to exercise caution before associating biochemical and other data with supposed morphogenetic phenomena. However, the mitoses and meioses occur only during particular periods of morphogenesis and not at all under certain conditions (Bonner and Frascella, 1952). Therefore, Bonner and Frascella (1952) suggest that morphogenetic movements are not dependent on mitoses occurring simultaneously.

This investigation is concerned with the nitrogen metabolism occurring in the slime mold and fragments of the slime mold during growth and morphogenesis. It is also the concern of this paper to associate these biochemical changes, insofar as possible, with morphological changes which occur during development of the slime mold.

METHODS FOR DETERMINING μG. N/μG DRY WT.[3]

The slime molds[4] were cultured according to the method of Bonner (1947). As individual slime mold pseudoplasmodia vary tremendously in size (at least 0.3–1.5 μg. total N) (Gregg, 1950) it was necessary to study nitrogen metabolism changes on the basis of dry weight. In order to weigh samples of slime mold tissue it was necessary to utilize a quartz helical balance having a range of from 1.0–1000.0 μg. The slime molds of various stages and fragments of pseudoplasmodia (Fig. 1) were placed upon tared bits of washed and dried cigarette paper (area approx. 12 sq. mm.). The slime mold tissues were dried in a vacuum desiccator

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[4] The cultures of *D. discoideum* were kindly provided by Dr. John Bonner, Dept. of Biology, Princeton University, Princeton, N. J.

TABLE I

Ranges of dry weight and nitrogen determined by analyses of whole and fragments of slime molds during the growth and morphogenetic stages

Type of determination	Vegetative amoebae	Migrating pseudoplasmodia	Mature sorocarps	Spores	Stalks
μg. dry weight	56.2–179.0	70.4–203.0	35.6–127.0	40.5–146.0	24.2–47.6
μg. nitrogen	5.35–18.0	4.40–20.8	3.03–11.6	3.38–14.6	0.38–2.96

at room temperature or in a drying oven at 60° C. for approximately twelve hours. The samples were then weighed on the quartz helical balance (Table I). The weighed samples of slime mold tissues were then analyzed for total N (TN). The magnitude of the dry weight and total nitrogen of the samples is shown in Table I.

The nitrogen determinations were made by a modification of the method of Bruel *et al.* (1946). The results were expressed as μg N/μg dry wt. Controls were conducted during the experiments by analyzing cigarette paper for the presence of traces of nitrogen.

GROWTH PHASE

Vegetative amoebae: Figure 1

The vegetative amoebae were prepared for analysis by the following steps:

1. Harvested from four Petri dishes by rubbing the agar surfaces with a glass rod in the presence of approximately 10 ml. of distilled H_2O. Amoebae and H_2O were filtered through a small bit of cotton to remove agar particles.

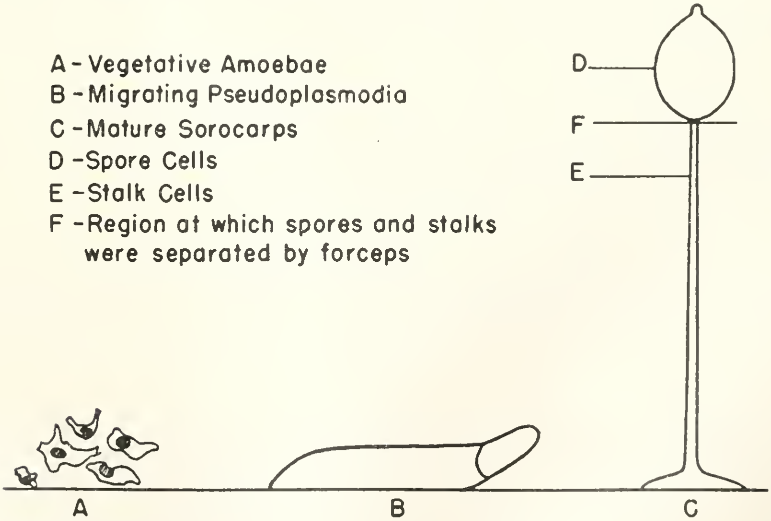
A - Vegetative Amoebae
B - Migrating Pseudoplasmodia
C - Mature Sorocarps
D - Spore Cells
E - Stalk Cells
F - Region at which spores and stalks were separated by forceps
D
F
E
A
B
C

FIGURE 1. Diagram of the growth and morphogenetic stages of the slime mold, *D. discoideum,* used in the analyses.

2. Amoebae centrifuged 5–10 minutes in a 15-ml. centrifuge tube at approximately 800 g.

3. Supernatant removed and discarded. The amoebae and *E. coli* at bottom of tube placed on surface of 10 ml. of 0.95 *M* sucrose solution in a 15-ml. centrifuge tube. Material centrifuged 5 minutes at approximately 500 g.

4. Supernatant removed and discarded. Amoebae placed in fresh tube of 0.95 *M* sucrose and centrifuged 5 minutes at approximately 500 g.

5. Supernatant removed and discarded. Amoebae suspended in distilled H_2O and recentrifuged 5 minutes at approximately 800 g.

6. Supernatant discarded and slime molds transferred to tared cigarette papers for drying, weighing and nitrogen analyses. The transfer was made with a thin-walled Pyrex glass pipette approximately 0.5 mm. I.D. at the tip.

Centrifugation of the slime mold amoebae and *E. coli* in 0.95 *M* sucrose tends to decrease the number of bacteria which are thrown to the bottom of the centrifuge tube. Upon microscopic examination of such preparations, very few bacteria were seen. It is believed that the relatively low numbers of bacteria present eliminate the possibility of significant interference by extraneous nitrogen in the analyses of the vegetative amoebae.

Vegetative amoebae subjected to the 0.95 *M* sucrose washing treatment, as described in steps 1–5, when placed on an agar surface aggregated and produced normal-appearing mature sorocarps.

MORPHOGENETIC PHASE

Migrating pseudoplasmodia: Figure 1

Between ten and twenty migrating pseudoplasmodia were transferred from the agar surface to the tared cigarette papers with a hair loop. The tissues were dried, weighed and analyzed for total nitrogen (TN).

Mature sorocarps: Figure 1

Between fifteen and twenty-five mature sorocarps were transferred from the agar surface to the tared cigarette papers with fine-tipped forceps. They were dried, weighed and analyzed for total nitrogen (TN).

Spores and stalks: Figure 1

The mature sorocarps were separated into spores and stalks with fine-tipped forceps and transferred to tared cigarette papers. They were dried, weighed and analyzed for total nitrogen (TN).

RESULTS

Total nitrogen (TN). Table II

The migrating pseudoplasmodia contain slightly less TN relative to their dry weight than the vegetative amoebae. This 9.13% loss is not statistically significant ($P > 0.05$).[5]

However, during the transition from the migrating pseudoplasmodia to the mature sorocarps, the TN/dry wt. ratio decreases 14.6% relative to that of the migrating pseudoplasmodia. Thus the mature sorocarps have been shown to contain significantly less TN/dry wt. than the migrating pseudoplasmodia ($P < 0.01$).

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[5] P values in this investigation were calculated by Student's t-test.

Analyses of the spores and stalks separately have shown that the observed loss of TN/dry wt. from the mature sorocarp did not come from the spores during culmination, since actually a slight increase (2.46%) occurred in the TN/dry wt. ratio of the spores, relative to that of the migrating pseudoplasmodia. This difference was not statistically significant ($P > 0.7$).

TABLE II

Analyses of nitrogen components of the vegetative and morphogenetic stages expressed as μg N/dry weight. These ratios represent the actual values with their standard deviations × 10^2. The dry weight values necessary in determining the final values of the various components other than total nitrogen were calculated from the total nitrogen/dry weight data. The numerals in parentheses refer to the number of experiments performed.

Nitrogen component	Vegetative amoebae	Migrating pseudoplasmodia	Mature sorocarps	Spores	Stalks
TN	10.30 ±0.691 (8)	9.36 ±1.52 (14)	7.99 ±0.650 (14)	9.59 ±2.94 (11)	4.73 ±1.55 (11)
TEN	5.70 ±2.47 (7)	6.81 ±0.730 (4)	4.49 ±0.976 (4)	6.20 ±2.80 (5	3.30 ±1.33 (4)
TEPN	4.75 ±2.61 (7)	5.66 ±0.260 (4)	2.81 ±0.957 (4)	2.63 ±0.656 (5)	0.987 ±0.333 (4)
TNPN	0.999 ±0.315 (7)	1.36 ±0.575 (4)	2.31 ±0.784 (4)	1.86 ±0.469 (5)	1.71 ±0.212 (4)
TUN	5.22 ±2.54 (7)	3.77 ±0.409 (4)	3.61 ±0.735 (4)	4.83 ±1.04 (5)	2.00 ±0.684 (4)
TUN + TEPN	9.97 ±1.46 (7)	9.43 ±0.641 (4)	6.42 ±0.509 (4)	7.46 ±1.06 (5)	2.98 ±0.776 (4)

TN = Total nitrogen
TEN = Total extractable nitrogen
TEPN = Total extractable protein nitrogen
TNPN = Total non-protein nitrogen
TUN = Total un-extractable nitrogen
TUN + TEPN = Total un-extractable nitrogen + total extractable protein nitrogen

The stalks, however, were shown to have lost a statistically significant amount of TN/dry wt. (49.5%) relative to the migrating pseudoplasmodia ($P < 0.001$).

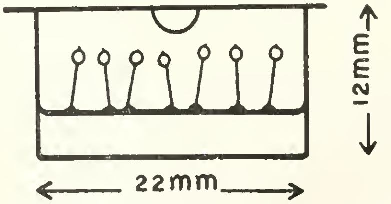

FIGURE 2. Diffusion cell with hanging drop of 0.01 N H_2SO_4 to absorb NH_3 liberated by the slime molds.

The loss of TN/dry wt. which was detected in the analyses of mature sorocarps relative to the migrating pseudoplasmodia may be attributed to an excretion of ammonia. The fact that ammonia is excreted by the slime mold *D. discoideum* in detectable quantities was determined by two methods.

The first method consisted in placing approximately twenty-four migrating pseudoplasmodia on non-nutrient or nutrient agar in a small Conway type vessel

Table III

Nitrogen (NH_3) excreted by D. discoideum *during the transition from migrating pseudoplasmodia to mature sorocarps determined by titration*

Mean no. of slime molds per diffusion cell	µg. N	µg. N—control	No. of experiments performed
24	—	0.545 ± 0.0153	3
0 (control)	0.009	—	1

(Fig. 2). A drop (11.8 µl.) of 0.01 N H_2SO_4 was placed on a paraffined glass slide, inverted and sealed to the vessel by means of a paraffin-vaseline mixture or Lubriseal stopcock grease. The slime molds were allowed to culminate completely. The drop of 0.01 N H_2SO_4 was then titrated to a methyl red end-point with a micro burette containing 0.01 *M* NaOH (Gregg, 1950) (Table III).

The second method was conducted similarly to the first with the exception that approximately 2.0 µl. of Nessler's reagent was added to the acid drop rather than titrating it. Nessler's reagent gave a strong positive reaction, demonstrating the presence of ammonia in the acid drop. Controls involving Nessler's reagent gave very weak positive results relative to the experimentals. These experiments have shown beyond doubt that ammonia is lost from the organism during certain of its developmental processes.

With a view to detecting possible changes in nitrogenous components which might be masked if total nitrogen analyses alone were considered, the various stages and fragments of the slime molds were fractionated in the manner described in Table IV. The fractions were designated as total extractable nitrogen, total extractable protein nitrogen, total non-protein nitrogen, total un-extractable nitrogen and total un-extractable nitrogen + total extractable protein nitrogen.

Table IV

Scheme designating source of various nitrogen components of slime mold tissues analyzed by micro-Kjeldahl procedure. (Modified from Gregg and Ballantine, 1946)

Slime mold tissues

|

Homogenize in extractant composed of 0.65% NaCl in 0.01 *M* phosphate buffer at pH 7.2 and dilute to 150 µl. with extractant

|

Remove 20–30 µl. for total nitrogen (TN)

|

Centrifuge remainder 15 minutes at 1000 g in Misco Air turbine

Particulate matter analyzed for total unextractable nitrogen (TUN)	Remove 30 µl. of supernatant for analysis of total extractable nitrogen (TEN)	Remove 80 µl. of supernatant. Deproteinize with 15 µl. of 0.67 H_2SO_4 followed by 8 µl. of 10% Na_2WO_4. Centrifuge 15 minutes at 1000 g in Misco air turbine. Analyze precipitate for total extractable protein nitrogen (TEPN). Analyze entire supernatant for total non-protein nitrogen (TNPN).

The slime molds were harvested from the agar surfaces by the techniques described previously (Steps 1–5) with the exception that the preparations were homogenized immediately on the ground glass surface of a micro-homogenizer (Fig. 3) rather than being subjected to a weighing procedure on the quartz helical balance. The magnitude of the nitrogen present in each of the five components is listed in Table V.

Total extractable nitrogen (TEN). Table II

The 12.0% increase in TEN/dry wt. of the migrating pseudoplasmodia was not shown to be statistically significant relative to that of the vegetative amoebae ($P > 0.3$). The TEN/dry wt. content of the mature sorocarps exhibited a 34.1% decrease from that of the migrating pseudoplasmodia. This difference is statistically significant ($P < 0.01$).

From the examination of the TEN/dry wt. values of the spores and stalks it may be seen that a 51.5% loss from the stalks constitutes the major part of the decrease noted in the mature sorocarps. This difference is statistically significant ($P < 0.01$) while the 8.96% difference in TEN/dry wt. between the spores and migrating pseudoplasmodia was not statistically significant ($P > 0.6$).

TABLE V

Ranges of nitrogen in various components determined by analyses of whole and fragments of the slime molds during the growth and morphogenetic stages

Nitrogen component analyzed	Vegetative amoebae	Migrating pseudoplasmodia	Mature sorocarps	Spores	Stalks
μg. TN	4.29–18.1	2.21–10.0	0.85–3.33	1.02–3.37	0.34–0.82
μg. TEN	1.43–19.8	4.13–12.1	2.28–4.69	0.89–5.87	0.27–0.75
μg. TEPN	2.59–47.1	11.0–20.9	2.89–6.49	0.89–3.26	0.27–0.75
μg. TNPN	1.45–5.50	2.0–6.33	1.60–6.36	0.57–2.46	0.56–1.11
μg. TUN	17.7–37.7	12.3–25.0	4.21–21.6	3.62–10.9	1.23–1.63

Total extractable protein nitrogen (TEPN). Table II

The 11.9% increase in TEPN/dry wt. which the migrating pseudoplasmodia exhibit relative to that of the vegetative amoebae is not statistically significant ($P > 0.4$). The mature sorocarps, however, show a significant decrease of 50.4% of their TEPN/dry wt. when compared to that of the migrating pseudoplasmodia ($P < 0.01$). The decrease occurs from both the spores and the stalks. Values of other nitrogen components of the spores, specifically, TN/dry wt., TEN/dry wt., and TUN/dry wt., suggested that little nitrogen utilization occurs in that region. The 53.5% decrease of the spore TEPN/dry wt. from that of the migrating pseudoplasmodia is statistically significant ($P < 0.001$) and is indicative of protein metabolism within the spores which has been masked in previous analyses by the presence of other nitrogenous components, namely TNPN/dry wt. and TUN/dry wt. The stalks show a decrease of TEPN/dry wt. amounting to 82.5% of the initial amount present in the migrating pseudoplasmodia. This difference is statistically significant ($P < 0.001$).

Total extractable non-protein nitrogen (TNPN). Table II

The value of the TNPN/dry wt. of the migrating pseudoplasmodia, although 36.1% greater than that of the vegetative amoebae, is not significantly different ($P > 0.1$). The mature sorocarps show a 69.9% greater TNPN/dry wt. content than the migrating pseudoplasmodia. Statistical analysis, however, fails to confirm what appears to be a significant difference ($P > 0.05$). The spores and stalks show increases relative to the migrating pseudoplasmodia of 36.8% and 25.7%, respectively. Neither of these increases is significantly different from the migrating pseudoplasmodia ($P > 0.1$ and $P > 0.2$). It is possible to compute the entire amount of non-protein nitrogen produced by making the assumption that the losses observed between certain of the TN/dry wt. values of the various stages resulted from an excretion of non-protein nitrogen. These values, in addition to the retained non-protein nitrogen values, comprise the entire quantity of non-protein nitrogen produced. Figure 4 demonstrates that the observed increase of the entire TNPN/dry wt. during development may be attributed to a quantitatively similar utilization of the TEPN + TUN/dry wt. component. The greatest discrepancy is found at the migrating stage since the level of TNPN/dry wt. is in excess of that which can be accounted for by a breakdown of the TEPN + TUN/dry wt. component. The excess TNPN/dry wt. can possibly be attributed to digestion of ingested *E. coli* with consequent production of non-protein nitrogen.

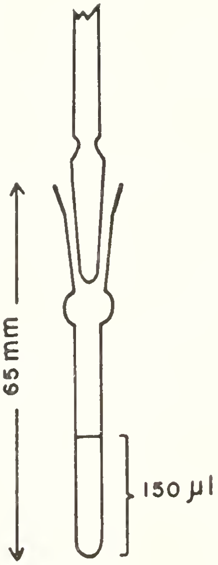

FIGURE 3. Micro-homogenizer constructed from a 5/20 Pyrex interconnecting joint.

Total un-extractable nitrogen (TUN). Table II

The TUN/dry wt. content of the migrating pseudoplasmodia shows a decrease of 27.8% relative to the vegetative amoebae. This decrease is not statistically significant ($P > 0.2$). The mature sorocarps show a 4.24% decrease as compared to the migrating pseudoplasmodia. Neither this decrease nor the 28.1% increase

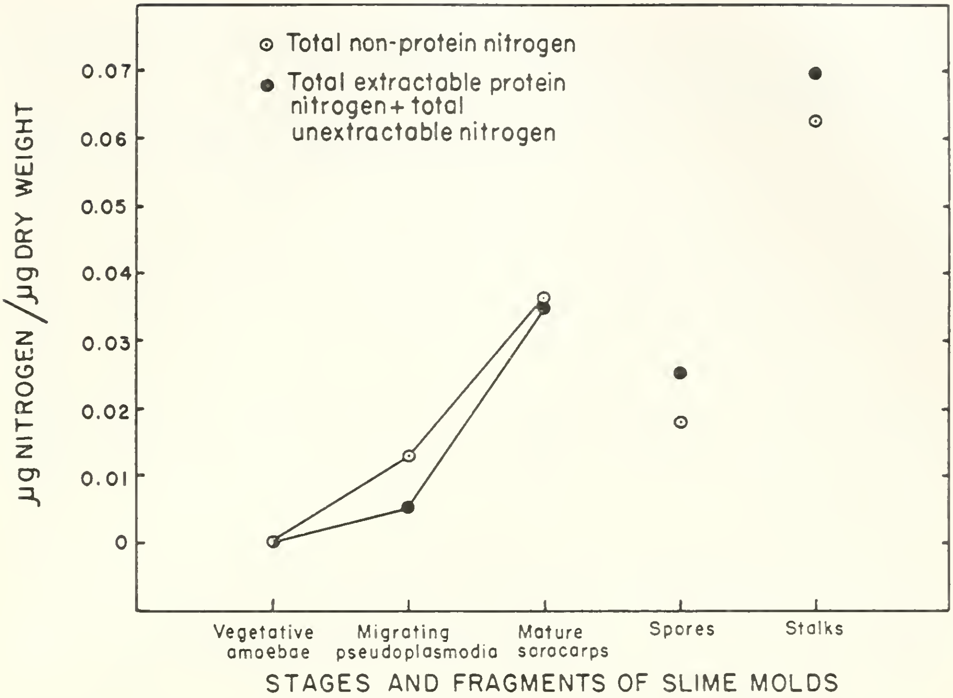

FIGURE 4. Graph relating the accumulated quantity of the TEPN + TUN/dry wt. component metabolized during development with the consequent increase in TNPN/dry wt. The accumulated TNPN/dry wt. was computed from the values of the retained TNPN/dry wt. plus that excreted from the organism as calculated from the decreases in TN/dry wt.

in the TUN/dry wt. component of the spores differs significantly from the value of the migrating pseudoplasmodia ($P > 0.7$ and $P > 0.05$). The slight but insignificant decrease in TUN/dry wt. indicated in the mature sorocarps results from the 46.9% decrease of TUN/dry wt. which occurs in the stalks. Relative to the migrating pseudoplasmodia this decrease has been shown to be statistically significant ($P < 0.01$). It appears as though this component is utilized by the stalks as well as the TEPN/dry wt. during the culmination of the slime mold.

Total extractable protein nitrogen + total un-extractable nitrogen (TEPN + TUN). Table II

When the sum of the two components TEPN/dry wt. and TUN/dry wt. was calculated it was found that the migrating pseudoplasmodia do not show a statistically significant decrease (5.42%) relative to the vegetative amoebae ($P > 0.5$). During culmination, however, a 31.9% decrease in the component TEPN + TUN/dry wt. occurred in comparison with the migrating pseudoplasmodia. This difference is statistically significant ($P < 0.001$). Both the spores and the stalks contributed to this decrease noted in the mature sorocarps. The decreases of the spores and stalks from the migrating pseudoplasmodia amounted to 20.7% and 68.4%, respectively. These differences are statistically significant ($P < 0.02$ and $P < 0.001$).

Discussion

Nitrogen changes occurring during the transition from the vegetative amoebae to the migrating pseudoplasmodia

The levels of the various nitrogenous components, total nitrogen/dry wt., total extractable nitrogen/dry wt., total extractable protein nitrogen/dry wt., total non-protein nitrogen/dry wt. and total un-extractable nitrogen/dry wt. of the vegetative amoebae and the migrating pseudoplasmodia were investigated. These values indicate that statistically significant nitrogen changes do not occur during the transition from the vegetative amoebae to the migrating pseudoplasmodia.

Nitrogen changes occurring during the transition from the migrating pseudoplasmodia to the mature sorocarps

The total nitrogen/dry wt. shows a statistically significant decrease during the transition from the migrating pseudoplasmodia to the mature sorocarps. The assumption has been made that this decrease reflects a loss of nitrogen resulting from the excretion of ammonia. The excretion of ammonia during this period has been demonstrated.

By a fractionation procedure it was possible to demonstrate that this loss of total nitrogen/dry wt. results from the metabolism of the total extractable protein nitrogen/dry wt. component. The increase in total non-protein nitrogen/dry wt. resulting from nitrogen metabolism during this period has been found to be quantitatively equivalent to the decrease noted in the total extractable protein nitrogen + total un-extractable nitrogen/dry wt. component (Fig. 4).

It was of interest to determine in which region of the pseudoplasmodium particular nitrogenous components were being utilized during the transition from the migrating stage to the mature sorocarp. In order that this might be accomplished, the mature sorocarps were separated into their major morphological entities, the spores and stalks. In analyzing the spores it was found that no loss of total nitrogen/dry wt. occurs. Therefore, the loss of total nitrogen/dry wt. observed in the intact mature sorocarps can be attributed to a decrease of total nitrogen/dry wt. in the stalks. The nitrogenous components responsible for the decrease of total nitrogen/dry wt. in the stalks were shown to be total extractable protein nitrogen/dry wt. and total un-extractable nitrogen/dry wt. The spores, too, show a decrease in total extractable protein nitrogen/dry wt., although of a smaller magnitude than that of the stalks. The spores do not show a loss in total nitrogen/dry wt. as simultaneous increases of other nitrogenous components are sufficient to mask the loss of total extractable protein nitrogen/dry wt.

From these data it is reasonable to suggest that both pre-spores and pre-stalks utilize proteins during the culmination process of morphogenesis. Since the prestalk cells are primarily responsible for raising the spore mass (Bonner, 1944; Raper and Fennell, 1952) it is not surprising that the metabolic requirements of the stalk-forming cells differ somewhat from those of the spore cells. During culmination the stalk cells build a cellulose sheath around themselves and cellulose is deposited in the walls of the spore cells (Raper and Fennell, 1952). It is suggested that the slime mold probably does not carry sufficient carbohydrate reserves both to synthesize cellulose and to utilize for purposes of obtaining energy. In this event proteins could be converted into cellulose or used for energy production.

While it is difficult to establish for which of these events protein metabolism is taking place, it is interesting to point out that the major nitrogen changes occur during the culmination process, at which time the spore and stalk cells are formed. While these data are suggestive of a relationship between protein metabolism and cellulose synthesis the hypothesis does not preclude the activity of other intrinsic mechanisms dependent upon protein breakdown.

Summary

1. Equipment and procedures incidental to determining the nitrogen metabolism of the vegetative amoebae, whole pseudoplasmodia, and fragments of pseudoplasmodia of the slime mold *Dictyostelium discoideum* during growth and morphogenesis have been described.
2. During the transition from the vegetative amoebae to the migrating pseudoplasmodia no statistically significant changes were found in any of the nitrogenous components under investigation.
3. During the transition from the migrating pseudoplasmodia to the mature sorocarps statistically significant decreases were found in certain nitrogenous components.
4. By analyzing spores and stalks separately it was possible to attribute nitrogen changes occurring in the intact mature sorocarps to particular regions.
5. The excretion of ammonia during the transition from the migrating pseudoplasmodia to the mature sorocarps was demonstrated.
6. The relationship between nitrogen metabolism and the synthesis of cellulose was discussed.

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EVIDENCE FOR A SWARMING SUBSTANCE[1] WHICH STIMULATES COLONY FORMATION IN THE DEVELOPMENT OF PEDIASTRUM DUPLEX MEYEN[2]

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The life cycle of the colonial, green alga *Pediastrum* has been known since the studies of Braun (1855), Askenasy (1888), Smith (1916) and Harper (1913, 1916, 1918a, 1918b). The colonies or coenobia of this organism are commonly found in fresh water and are disk-shaped in form. The cells of the colony are arranged in a single layer with the marginal cells usually differing from those of the interior by possessing one or two prongs or processes produced radially outward. The number of cells in a colony varies but in general, can be described by the simple formulation 2^N, the common range being eight to sixty-four. Asexual reproduction involves the formation of biflagellate zoospores which arise through successive nuclear divisions, followed by progressive cleavage of the cell chloroplast. After the zoospores have been formed they are discharged through a crescent-shaped slit in the mother cell wall and are enclosed in a thin, transparent sac or vesicle. At first, they form an irregularly shaped mass, but after three to four minutes of active movement, they slow down forming a flat plate of cells. Complete cessation of movement then follows and prong formation begins and is completed in a matter of minutes.

Although details of the life cycle have long been known, little work has been done concerning the factors affecting the production of zoospores and their release to form new colonies. The purpose of the study presented in this paper was to examine the characteristics of growth and reproduction in *Pediastrum* when grown in culture and to investigate the role of certain factors which influence these processes. The results will be concerned with such aspects as the time at which colony formation begins and how its initiation is affected by pH and aged medium. Evidence will be presented for the occurrence of a substance or group of substances in aged medium which can alter the time at which colony production begins, the number of colonies produced and the size of these colonies in terms of cell number.

Materials and Methods

The species of *Pediastrum* used in this study was *P. duplex* Meyen (from Malham) and was provided by E. G. Pringsheim of the Cambridge University Botany School in unialgal, non-bacteria-free culture. It was grown in this form on mineral medium No. 8 according to Chu (1942) with 10% soil extract added. All of the cultures used in this section were prepared under sterile conditions and grown in Ehrlenmeyer flasks.

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[1] The word "substance" is used to denote one or more substances active in stimulating colony formation in *Pediastrum duplex* Meyen.

[2] This paper represents a part of a dissertation in Biology presented to the faculty of Princeton University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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For most of the experiments the culture vessels were placed in a trough with tap water circulating through it to maintain a relatively constant temperature. The lighting was provided by two 15-watt daylight General Electric "fluorescent" bulbs set parallel 26 cm. apart in a wooden housing and elevated 25 cm. above the culture-containing trough. In one experiment only (Section A), the culture was not grown in a water-circulated trough but was simply placed on a wooden platform and allowed to grow at room temperature (24–28° C.). This culture was illuminated by a 100-watt tungsten filament bulb housed in a desk-type lamp and placed 40 cm. in front of the culture. In all cases the lighting was continuous throughout the course of the experiment. It should be noted that temperature was not continuously recorded by any mechanical device but that periodic measurements were made daily using a standard mercury thermometer. When the cultures were grown in the water-circulated trough the temperature of the circulating water was determined; when a culture was grown in the open air the temperature of a flask of water placed next to the culture flask was determined. The constancy of the light source (*i.e.*, intensity and wave-length) was also not strictly controlled. It was felt by the author that the aim of this investigation did not necessitate the use of rigid controls of temperature and light since in every experiment both control and experimental cultures were grown under identical conditions. Any investigation of the effects of temperature and light on the phenomena reported in this paper would, of course, require strict control of these two factors and it is hoped that a study of this nature will be possible during the course of future work.

For counting the number of colonies in a culture, two counting chambers were used: The Levy hemacytometer and the Sedgwick-Rafter counting chamber. For determining colony number with the Levy hemacytometer the method for counting leucocytes was used according to the following formulation:

$$\text{Colonies/cc.} = \frac{\text{Colonies counted} \times \text{dilution} \times 10^4}{\text{No. of 1 sq. mm. areas counted}}$$

To determine colony number with the Sedgwick-Rafter counting chamber the number of colonies in one pathway was counted using a 10 × objective and 20 × ocular and the following formulation used:

$$\text{Colonies/cc.} = 17 \times \text{colonies in one pathway} \times \text{dilution} \times 2.$$

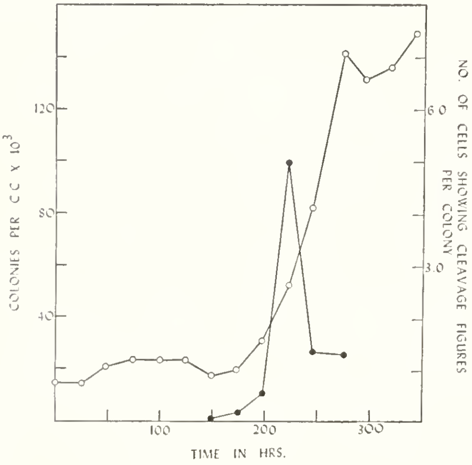
The colonies were always fixed in 2–3% formalin before counting. In all experiments but one the Sedgwick-Rafter chamber was used to determine colony number.

To determine cleavage number the colonies were fixed in formalin-aceto-alcohol and stained with Harris' hematoxylin according to the method of Johansen (1940). A drop of suspension containing stained colonies was then placed under a cover slip, sealed with paraffin and the number of cells showing cleavage figures was recorded for 150 colonies counted. To make these counts the 95 × oil immersion objective and 10 × ocular were used. pH measurements were made with a Cambridge pH-Meter Laboratory Model-L.

RESULTS

A. Quantitative relations of growth and colony production

At the outset of this work on *Pediastrum* it was desirable to obtain large numbers of swarming colonies in a relatively short time. To accomplish this, large inocula of 5 cc. for every 50 cc. of fresh medium were used in preparing cultures. When this was done it was noticed that swarming occurred in great numbers at a specific time after inoculation and that it seemed to last for only a few days. The time that swarming or colony production began could be predicted with reasonable accuracy in succeeding cultures inoculated in the same manner from the same stock. Virtually no swarming was found in the cultures prior to this swarming period and after it was completed.



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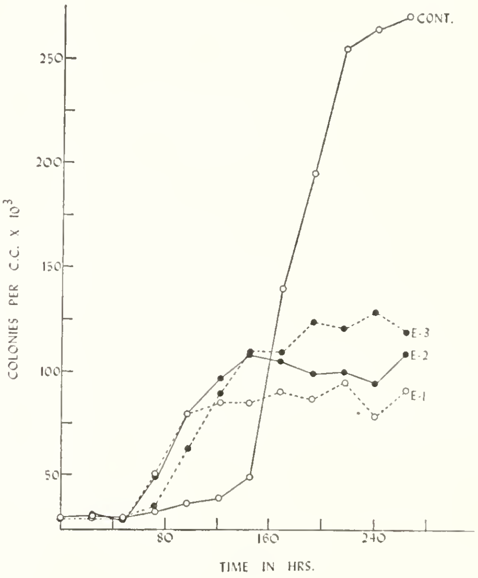

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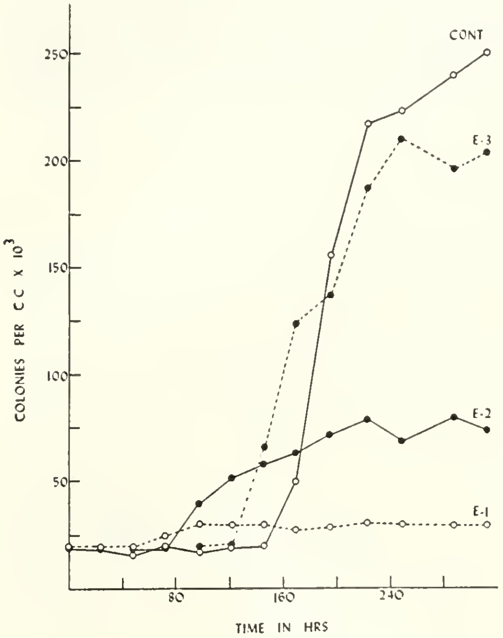
FIGURE 1. Graph showing colony production and cleavage activity curves. Open circles represent colony production and black dots represent cleavage activity.

FIGURE 2. Graph showing colony production in fresh or control medium and in aged medium after 0 (E-1), 1 (E-2) and 2 (E-3) days' growth in fresh or control medium.

In order to obtain a quantitative description of colony formation as a function of time the following experiment was performed: a culture was started consisting of 50 cc. of fresh medium inoculated with a 5-cc. suspension of 99-day old stock culture and grown in a 125-cc. flask. The lighting was provided by a 100-watt tungsten filament bulb and the experiment run at room temperature (24–28° C.; see Materials and Methods). One-cc. aliquots were removed daily from the culture, fixed and colony number determined using a Levy hemacytometer chamber. In the results in Figure 1 it is seen that no colony production occurs in the culture up to 173 hours but that a sudden burst begins at 197 hours and lasts for 78 hours at which time it begins to level off. The final number of colonies is approximately 8 times the value of the starting colonies which is what would be expected if the original mean colony size was 8 cells (see Discussion). Cleavage counts were made to see whether cleavage activity coincided with the burst phase of the curve. In the graph of Figure 1 this activity is expressed as the number of cells showing cleavage figures per colony and it can be seen that there is a correlation between colony formation and cleavage activity. It should be noted that although no swarming takes place in the culture up to 173 hours, considerable growth, in terms of colony size, occurs during this period.



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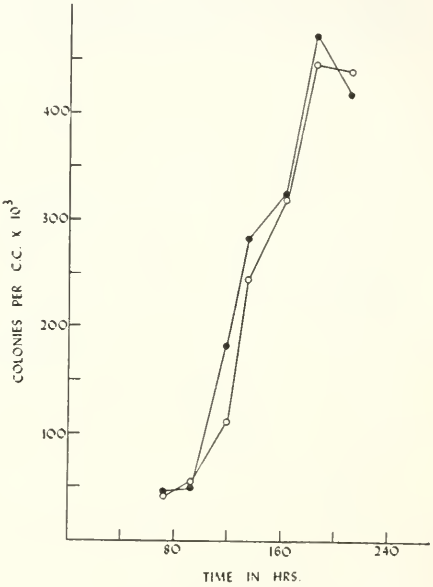

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FIGURE 3. Graph showing colony production in fresh or control medium and in aged medium after 0 (E-1), 2 (E-2) and 4 (E-3) days' growth in fresh or control medium.

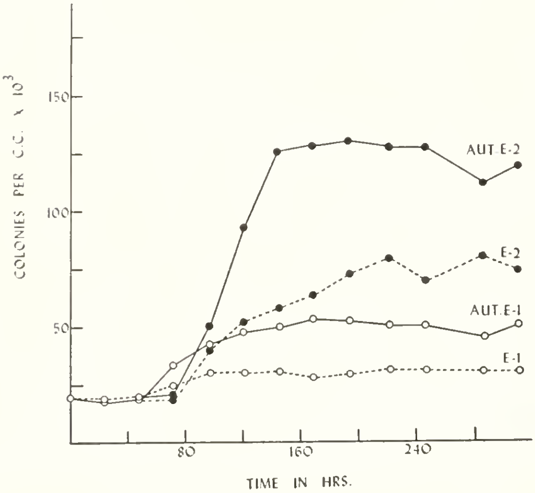
FIGURE 4. Graph showing results obtained when colonies are placed in aged medium one day prior to the time they will swarm in fresh or control medium. Open circles represent colony production in the control and black dots represent colony production in the experimental.

B. Evidence for a substance in the aged medium affecting colony reproduction

The curious nature of the swarming curve suggested the possibility of some change occurring in the medium which might initiate swarming or colony formation. Experiments were performed to test how colony production was affected by moderately aged medium, in which swarming had occurred. In the first experiment 150 cc. of fresh medium were inoculated with 15 cc. of a 53-day old stock culture and placed in a 500-cc. flask to serve as standard culture. Aged medium was obtained from a 19-day old culture from which the colonies had been removed by centrifugation. Using the standard culture and aged medium the following cultures were prepared:

Control: 30 cc. of standard culture. Prepared at zero time.
Experimental No. 1: Colonies from 30 cc. of standard culture, fresh medium removed and replaced by 30 cc. of aged medium. Prepared at zero time.
Experimental No. 2: Colonies from 30 cc. of standard culture after one day's growth, fresh medium removed and replaced by 30 cc. aged medium.
Experimental No. 3: Colonies from 30 cc. of standard culture after two days' growth, fresh medium removed and replaced by 30 cc. aged medium.

One-cc. aliquots were removed daily from all the cultures and colony number determined. The temperature maintained throughout the course of the experiment was 20 ± 2° C. In the results shown in Figure 2 it is seen that the control shows the familiar swarming burst after 144 hours but that all three experimentals show a substantial increase in colony number after 73 hours. The longer the colonies grow in fresh medium, the greater the level of colony production reached after they are transferred to aged medium. None of the experimental cultures produce as many new colonies as the control.



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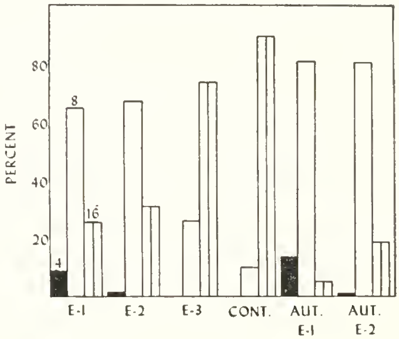

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FIGURE 5. Graph showing results obtained when colonies are grown in unautoclaved and autoclaved aged medium after 0 (E-1) and 2 (E-2) days' growth in fresh or control medium.

FIGURE 6. Graph showing the final percentage distribution of four-, eight- and sixteen-celled colonies produced in the control and the unautoclaved and autoclaved aged medium cultures of Figures 3 and 5.

In a repeat experiment similar to the preceding one colonies were allowed to grow in fresh or control medium for 0 (Exp. No. 1), 2 (Exp. No. 2) and 4 (Exp. No. 3) days before being transferred to aged medium. The standard culture was prepared from a 58-day old stock culture and the aged medium was obtained from a 15-day old culture. In the results shown in Figure 3 it is apparent that all three experimental cultures show colony production before the control does. Once again, the longer the colonies grow in fresh or control medium the greater the number of new colonies they produce when transferred to aged medium. The colonies grown for 4 days in fresh or control medium prior to transfer to aged medium show a final level of colony production very close to that of the control.

Figure 4 shows the results obtained when colonies, one day prior to the time they will normally begin swarming, are placed in 8-day old aged medium. In this experiment the colonies had been growing three days in fresh medium when they were placed in aged medium. The standard culture was prepared using 87-day old stock culture and the temperature maintained throughout the course of the experiment was 19 ± 2° C. It is seen that although both cultures begin swarming at the same time the experimental shows a greater burst. Essentially the same final level of colony production is reached in both cultures.

TABLE I

Final distribution of colony types produced in the control and experimental cultures of Figures 3 and 5.

Culture	4-celled colonies		8-celled colonies		16-celled colonies	
	New colonies/cc.	% of total	New colonies/cc.	% of total	New colonies/cc.	% of total
E-1	1,112	8.52	8,588	65.8	3,356	25.7
E-2	756	1.52	33,568	67.6	15,331	30.8
E-3	0	0	50,837	26.0	144,983	74.0
Control	0	0	20,624	9.95	186,497	90.0
Aut. E-1	4,427	13.9	25,826	81.2	1,571	4.94
Aut. E-2	1,163	1.17	80,294	80.5	18,403	18.4

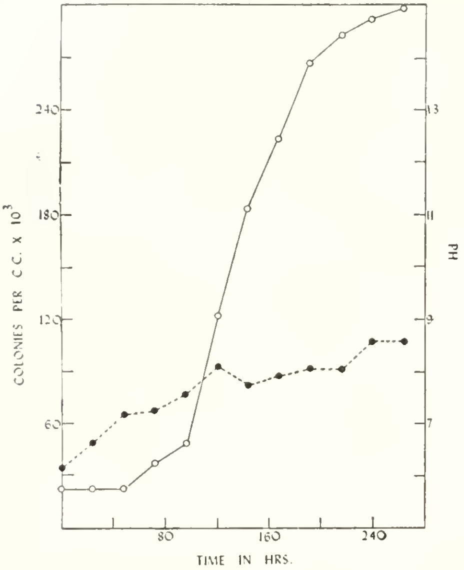
C. The effect of high temperature on the activity of the aged medium

To test the effect of high temperature on the activity of aged medium the following experiment was performed: Aged medium used in the second experiment reported in section B was autoclaved for 30 minutes under 15 lbs./in.^{2} pressure at 121° C. Colonies were placed in this autoclaved aged medium after 0 and 2 days growth in fresh medium and the cultures were run simultaneously with Experimentals No. 1 and No. 2 of the second experiment reported in section B, both of which contained unautoclaved, aged medium. From the results shown in Figure 5 it is evident that the two autoclaved experimentals show colony production at the same time as the unautoclaved counterparts. However, the colonies placed in autoclaved aged medium after 0 days growth in fresh medium produce 3 times as many new colonies as the unautoclaved partner, while those placed in autoclaved aged medium after two days' growth in fresh medium produce almost twice as many new colonies as the unautoclaved partner.

Table I and Figure 6 show the per cent distribution of 4-, 8- and 16-celled colonies produced by the control and three experimental cultures of the second experiment reported in section B and the two autoclaved aged medium experimentals of this section. The results show that the longer the colonies are kept in the control medium before transfer to aged medium, the greater the tendency to produce colonies with larger cell number.

D. The role of pH of the medium

Two experiments were performed to determine whether the effect of aged medium in causing premature colony formation was due to pH change in the medium. Since the medium is poorly buffered it was thought that pH change might be an influencing factor. A culture was started consisting of 200 cc. of fresh medium inoculated with 20 cc. of 56-day old stock culture and grown in a 500-cc. flask. Two (5-cc.) aliquots were removed daily and pH and colony number determinations made. The temperature maintained throughout the course of the experiment was 19 ± 2° C. Figure 7 shows the results obtained. It is clear that change of pH from the acid to the basic side occurs during the growth and reproduction of *Pediastrum* under these culturing conditions; pH changed from an initial 6.13 to a final 8.60 but there does not appear to be any sudden increase in pH



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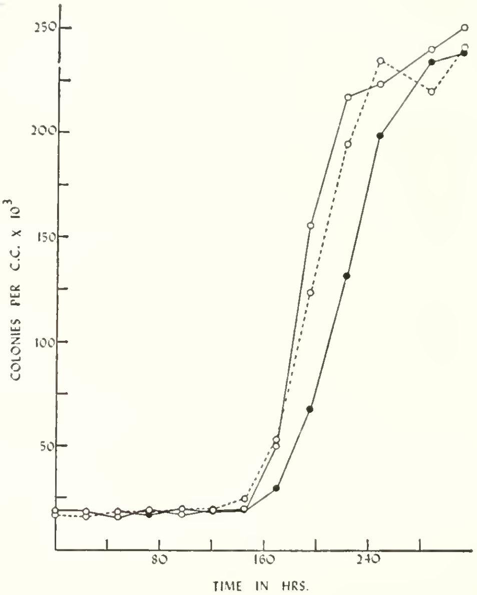

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FIGURE 7. Graph showing curves of colony production and pH change of the medium during colony production. Open circles represent colony production and black dots represent pH change.

FIGURE 8. Graph showing results obtained when colonies are grown in pH-regulated medium (7.31) after 0 (E-1) and 2 (E-2) days' growth in fresh or control medium. Open circles connected by continuous line represent colony production in the control; open circles connected by dotted line represent colony production in pH-regulated E-1 and black dots represent colony production in pH-regulated E-2.

at the time of colony formation. Instead a gradual, continuous increase occurred both prior to and during this phase.

Since pH change did occur in the medium during the development of *Pediastrum* another experiment was performed to test whether this change was the causative influence in the premature swarming effect of aged medium. In this experiment th pH of fresh medium (6.29) was regulated with 0.1 N NaOH to that of the aged medium used in the second experiment of section B (7.31). Colonies were placed in this pH-regulated medium after 0 and 2 days' growth in fresh, unregulated medium and the culture was run simultaneously with the control and Experimental No. 1 and No. 2 of the second experiment of section B. In the results shown in Figure 8 it is seen that both pH-regulated cultures show the swarming burst at approximately the same time as the control. The colonies receiving the pH-regulated medium after two days' growth in fresh unregulated medium apparently respond somewhat later than those of the control. When the experiment was completed the pH of the control was 7.51, the 2-day aged medium experimental (Exp. No. 2) was 7.50 and the 2-day pH regulated experimental was 7.72.

Discussion

It has been shown for the first time that colonies of *Pediastrum* when grown in cultures using large inocula in fresh media exhibit two developmental phases. The first is a latent period which may last for 3–7 days, in which the colonies grow but do not reproduce. The second phase is a very active period of swarming or colony production which lasts 3–4 days during which all the cells in the initial inoculum give rise to new colonies. Cleavage activity, as would be expected, has been shown to be quite synchronous with this period. That essentially all the cells in the original colonies give rise to new colonies can be demonstrated by determining the average number of cells per colony in the inoculum and multiplying this value by the number of colonies present. In the experiment concerning colony type distribution the control showed an average colony size of 14.0 cells/colony and a colony number of 18,718/cc. in the starting culture. If all the cells gave rise to colonies then the value reached by the control after swarming should be (14.0) (18,718) or 262,052/cc. The actual value reached by the control is reasonably close: 252,450/cc.

The fact that cultures exhibit a latent period might have initially been interpreted as some inherent time lag in the ability of the colonies to reproduce during this period. However, the results show that by placing colonies in medium in which swarming has occurred colony formation can be induced prematurely. The longer the preliminary period of growth in control or fresh medium, the greater the number of colonies produced after transfer to aged medium and the more closely the resulting swarming curve resembles that of the control. Coincident with these changes is the gradual increase in colony size in terms of cell number as the preliminary period of growth in control or fresh medium is lengthened. Again, the longer this preliminary period of growth the more closely the colony type distribution resembles that of the control. It appears evident that the latent period in the normal swarming curve of *Pediastrum* is one in which the competence of the colonies to reproduce is ever increasing. This might conceivably be a function of critical size whereby colonies which are too small and immature to reproduce are transformed, during the period of growth, into colonies of adequate size. The longer this period of growth the more abundant the number of colonies reaching reproductive maturity. The fact that colonies can reproduce during the latent period, and yet do not, indicates that the proper stimulus for reproduction is not present at this time. The evidence indeed indicates that the swarming burst is a response to some substance or substances accumulating in the medium during the growth of the colonies and that the latent period is simply an index of the time required for the substance to reach a threshold concentration. That the action of this substance is not through a pH effect has been shown by placing colonies in medium the pH of which has been regulated to that of aged medium and yet the pH-regulated medium has no stimulating effect but parallels normal control medium. The substance is not only heat-stable, but for some reason, has its activity enhanced by high temperature. This increase in activity could conceivably be caused by the removal or breakdown of inhibitory factors present in the medium, or to the production of breakdown products which can further induce colony formation.

The origin of the swarming substance cannot be determined unequivocally from these experiments. The possibility of bacterial origin exists and can only be ruled out through repeat experiments with pure cultures.

It is known, however, that certain algae do produce substances capable of influencing growth. Lefevre, Jacob and Nisbet (1952) in their studies on various fresh water algae have found evidence for the production of substances which can inhibit not only the growth of the algae producing them (autoantagonism) but the growth of other species of algae as well (heteroantagonism). Extensive work with *Pandorina* and *Scenedesmus* has revealed the production by these organisms of inhibitors which the authors have named Pandorinine and Scenedesmine, respectively. These substances, in addition to being autoantagonistic in their inhibitory power, will inhibit the growth of several other algae including various species of *Pediastrum*. It is indeed interesting that boiling the inhibitor usually lowered its inhibitory effect and, in some instances, actually caused it to give an enhancement of growth. This, in some respects, parallels the experiments presented in this paper regarding autoclaving of the swarming substance. It seems quite possible that at least some algae produce both growth inhibitory and stimulating substances as a normal part of their metabolism and that the former are more heat-labile than the latter.

If the swarming substance is produced by the *Pediastrum* colonies then it is most interesting to review the results of Smith (1916) who studied *P. Boryanum* in pure culture. Smith found that for the first two weeks in culture 64- and 32-celled colonies were predominant while a month later 8- and 16-celled colonies were most common. A reasonable explanation of these observations is now available. Since pure cultures in general are prepared with very small inocula it seems likely that Smith's cultures contained small numbers of starting colonies. If growing colonies were producing a swarming inductor it would take a considerable length of time for a small number to produce enough substance to initiate reproduction. The colonies would be expected to grow to a rather large size before a threshold concentration of the substance was reached and colonies with large cell numbers would be produced. However, as the culture grew older new colonies could not grow as long for the substance would be present tending to induce reproductive activity and colonies of fewer cell number would be produced.

The role of such a substance in the free-living *Pediastrum* colony in nature would be most important. In an actively moving, nutrient-rich environment colonies could grow for an extended time without the substance collecting. In this manner maximum growth would take place and when reproduction finally occurred colonies of great cell number would be produced. In an inactive, stagnant environment, however, the substance would tend to build up and finally force reproduction. It is easy to see the survival value of an increase in numbers for dissemination to more favorable conditions. To speculate even further, it is not difficult to imagine that this swarming substance could, at the proper concentration, stimulate sexual reproduction and the formation of free-swimming gametes. There are some indications that this might be so. It is known, from the author's experience, that when concentrated suspensions of swarming colonies are left standing for a day or more, free-swimming gamete-like cells are often produced. Since fusion between these cells has never been observed it is not known for certain whether they are gametes. Askenasy (1888) found that he could initiate gamete production sooner in his cultures if the colonies were kept in a smaller volume of water. If colonies were living in a drying pond the substance would build up and finally force gamete production. This mechanism would have the advantage of bringing the sexes together in concentrated form, thereby facilitating the meeting of the sexes and fusion. Furthermore, the result of fertilization is a resistant polyhedron which is capable of surviving the drought and germinating again when the rains come and the pond fills up. This hypothesis concerning the role of the swarming substance in sexual reproduction should be capable of testing and experimental work on this subject is planned.

The author wishes to thank Dr. John Tyler Bonner for his fruitful guidance and encouragement during the course of this work.

SUMMARY

1. The characteristics of growth and reproduction in *P. duplex* Meyen have been studied in non-bacteria-free cultures using large inocula of 5 cc. of stock culture for every 50 cc. of fresh medium.

2. It has been found that colonies grown under these conditions exhibit two phases of development: A latent period in which no colony production or swarming takes place but during which the colonies grow, and a period of active swarming or burst phase during which all the cells of the starting colonies give rise to new colonies.

3. It has also been shown that a heat-stable substance is present in aged medium which can, through an effect other than pH, alter the time at which colony production begins, the number of colonies produced and the size of these colonies in terms of cell numbers.

4. It is suggested that the length of the latent period of development is determined by the time required for this substance to reach a threshold concentration. The possible importance of such a substance to free-living forms of *Pediastrum* in nature is also discussed.

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SECRETION OF GASES AGAINST HIGH PRESSURES IN THE SWIMBLADDER OF DEEP SEA FISHES.

I. OXYGEN DISSOCIATION IN BLOOD[1]

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By studying the composition of the swimbladder gases in relation to depth it was established that both oxygen and nitrogen are deposited into the swimbladder against a concentration gradient to make up the hydrostatic pressure. The nitrogen is deposited as a certain fraction of the oxygen varying from 2 to 15%, according to the species. Carbon dioxide and organic gases contribute only insignificantly to the pressure. The oxygen pressure commonly reaches values above 100 atmospheres, and it is likely that it can be two to three times as much, as species with swimbladders are known to occur down to a pressure of at least 266 atmospheres. The nitrogen pressure in deep sea species frequently reaches 10–15 atmospheres (Scholander and van Dam, 1953).[2] In whitefish (*Coregonus*) pure nitrogen may be deposited into the swimbladder against 6–8 atmospheres' pressure (Hüfner, 1892). Saunders (1953) has verified this finding, and has added four more species of physostome fresh water fishes which also seem to deposit nothing but nitrogen in the swimbladder.

Current Theories of Gas Secretion

Haldane (1922) in his celebrated book "Respiration" gives his views as to a possible mechanism for the gas secretion, which in its basic concepts expresses the ideas offered by later authors. He saw in the *rete mirabile* a counter current exchange mechanism (p. 214): "The arrangement reminds us of that in a regenerating furnace, where the heat carried away in the waste gases is utilized to heat the incoming air." As to the chemical release of oxygen he points out the importance of CO_2 in expelling oxygen from the hemoglobin and states (p. 215): "It seems probable, therefore, that the function of the *rete mirabile* is to enable venous blood to communicate part of its CO_2 to the arterial blood. The effect of

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[1] Contribution No. 699 from the Woods Hole Oceanographic Institution. This investigation was supported by a grant from the National Science Foundation.

[2] In our 1953 paper data are given on gas samples from *Alepocephalus* and *Cottunculus*. From the excellent review of Jones and Marshall (1953) we learned that *Alepocephalus* does not have a swimbladder and this we verified on our recent material. We found also that *Cottunculus* has no swimbladder. This raises the question as to where the gas came from of which we obtained a few cubic centimeters by poking around in these fishes with our needle. The presence of the gas was obvious because the fishes were floating on the water. The composition of this gas checked well with that of the other fishes in the same catch, and hence it is believed that the fish must have swallowed some of the gas gushing out from other fishes when the trawlbag approached the surface. Obviously a fish distended by swimbladder gas cannot pollute this gas by swallowing.

this will be to raise the CO_2 pressure of the blood supplied to the gland, and so raise the oxygen pressure." Already Müller (1840) and Woodland (1911), who presented accurate anatomical drawings of the *rete*, had considered that they must be interpreted as a diffusion apparatus between the arterial and venous capillaries.

Hall (1924) found that, if the gas gland in yellow perch (*Perca flavescens*) was stimulated to activity by removing gas from the swimbladder or by increasing the hydrostatic pressure, a dialysate of the active gland showed a decrease in pH from 7.1 to 6.4, as compared with a non-active gland. He assumed secretion of an acid by the glandular epithelium. This would diffuse into the capillaries of the *rete* and by releasing bound oxygen would raise the oxygen tension, permitting the oxygen to diffuse into the swimbladder. If the oxygen capacity were 9 vol. % and the solubility coefficient 3 vol. % per atmosphere, then this mechanism could raise the oxygen tension no more than 3 atmospheres. This would be ample for shallow water fish like the perch, but could not explain the secretion of 100 atmospheres or more in deep sea fishes.

Jacobs (1930) found a way out of this difficulty by elaborating somewhat further on the possible function of the *rete mirabile*. When acid is secreted by the glandular cells and diffuses into the blood, CO_2 is released, increasing the CO_2 tension. The CO_2 effect itself, or possibly the secreted acid, dissociates oxygen from the oxyhemoglobin, so that both CO_2 and oxygen tension increase. This would explain the fact that both O_2 and CO_2 tension increase when gas is generated following a puncture. The blood with elevated O_2 and CO_2 tension leaves the gland and enters the efferent (venous) *rete*, where the gases diffuse over to the afferent (arterial) side, boosting the tensions there. When this high tension blood enters the gland, new acid is added, the tensions are again stepped up, and so on, until high enough tension has been built up in the swimbladder.

The secretion of oxygen would accordingly involve two principal steps: (1) a chemical dissociation of oxygen by acid (and/or CO_2) from the oxyhemoglobin, and (2) a counter current exchange mechanism (the *rete mirabile*) which would retain the secreted gases within the swimbladder with a minimal loss to the outside, in spite of maintained circulation. These basic ideas have been accepted by almost all later workers as the likely mechanism for oxygen secretion in fishes (Powers *et al.*, 1932; Koch, 1934; Irving and Grinnell, 1939; Black, 1946; Copeland, 1952; Strittmatter, Ball and Cooper, 1952; Fänge, 1953). Von Ledebur (1937) expressed serious doubts as to the correctness of the chemical interpretation.

In the present account we shall deal with the oxygen dissociation at pressures from 0.2 to 140 atmospheres and its relation to pH and CO_2. The function of the *rete mirabile* will be dealt with in a later account.

Oxygen Dissociation in Blood from Deep Sea Fishes in Relation to pH and CO_2

The chemical or physical reaction which is in the end responsible for the oxygen deposition in deep sea fishes must be able to perform against a pressure which at least equals the partial pressure of oxygen known to occur in the fish in question. Many deep sea fishes secrete oxygen against 100 atmospheres or more, and by inference some secrete against considerably more than 200 atmospheres.

It has long been known that the blood of many fishes possesses a large Bohr effect (Krogh and Leitch, 1919, and many later authors). Root (1931) and Green and Root (1933) found that in several fishes the blood did not become completely saturated if acid or CO_2 was added at oxygen pressures up to near one atmosphere. The saturation curves were found to run virtually horizontal at pressures above 0.5 atmosphere. The *Bohr effect* is generally considered a change in the dissociation constant (K in the Hill equation) with 100% saturation obtainable at high enough pressure. The term *Root effect,* on the contrary, may be reserved for the situation where acidity completely blocks the oxygen from part of the hemoglobin.

Obviously, before we can know whether the Bohr or Root effect can be responsible for the oxygen secretion against 100–200 atmospheres' pressure, we must extend the dissociation curves to these pressures. This has been done for a series of ocean fishes inhabiting depths equivalent to pressures from 40 to as much as 260 atmospheres.

The investigation was commenced in the winter of 1952–1953 at the Lerner Marine Laboratory, Bimini, Bahamas, and was amplified by material caught on the dragger *Cap'n Bill II* during two cruises in the summer of 1953 off the New England coast.

Material

Fishes were caught at the bottom by hook and cable from 400–700 meters' depth in the Gulf Stream off Bimini between December, 1952 and February, 1953. For this purpose a gasoline-powered fishing rig was used, originally designed by Captain Eddie Wall at the Lerner Laboratory. The fishes were brought to the surface within 4–7 minutes after hooking, according to the depth. Blood was drawn immediately by hypodermic needle from the heart and transferred to a polyethylene bottle containing a little heparin. The sample, of about 20–50 cc., was stored on ice and brought to the laboratory within an hour. The blood was then put through all the necessary procedures at once.

Three species were obtained at Bimini:

			Depth of catch
3	*Epinephelus mystacinus*	Black grouper	320–400 m.
1	*Alphestes* sp.[3]	"Golden grouper"	410 m.
2	*Polyprion americanus*	Wreckfish	640 and 675 m.

The fishes weighed between 10 and 30 kg. each.

Deep sea material was likewise obtained on board the dragger *Cap'n Bill II,* operating from Woods Hole, on two cruises (June 16–23 and July 1–8, 1953). The fishes were caught by bottom trawl, mostly at depths of between 500 and 1300 meters, depending upon the species. It took up to three quarters of an hour to get the trawl to the surface from the time it left the bottom. The blood could in some cases be drawn from the heart (*Sebastes*). In other cases blood was obtained which dripped from the cut tail. It was usually necessary to pool blood from many specimens to get enough for the analyses, which were performed immediately.

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[3] Called *Epinephelus,* Grouper, in Scholander and van Dam (1953).

The following species were caught and analyzed on the *Cap'n Bill:*

		Depth of catch
Antimora viola	Blue hake	1200 m.
Coryphaenoides rupestris	Round-nosed ratfish	1100 m.
Sebastes marinus[4]	Rosefish	550– 720 m.
Synaphobranchus pinnatus	Long-nosed eel	960–1100 m.
Urophycis chesteri	Long-finned hake	600–1300 m.

Optical method for determination of dissociation curves at high pressures

The problem required a study of oxygen dissociation of fish blood at different pH or CO_2 tensions carried from an oxygen pressure of 0.2 atmosphere, if possible, up to values corresponding to the greatest depth at which the species was known to occur. As a routine the highest oxygen pressure employed was that of a fully charged commercial gas cylinder, *i.e.,* 140 atmospheres.

An earlier survey (van Dam and Scholander, 1953) showed that the blood of deep sea fishes had about the same oxygen combining capacity as that of shallow fishes, *i.e.,* about 6–10 vol. %. At 100 atmospheres' pressure the blood would physically dissolve about 300–400 vol. % oxygen, and hence it would be impossible by direct gasometric methods to determine the saturation of the hemoglobin. An optical method was therefore designed.

The method must be accurate to within a few per cent saturation, and it must be possible to operate it on a rolling ship. It was necessary to equilibrate the blood sample in the photometer cuvette, which must be accurately controlled to the same temperature as that at which the bottom fishes live. The cuvette must be able without significant distortion to stand a pressure of 140 atmospheres.

As a measuring instrument a Zeiss Pulfrich Stufen Photometer[5] was employed. The construction of the high pressure cuvette will be seen in Figure 1. The glass windows were ½-inch thick and the effective depth of the blood in the optical paths was 1.6 mm., increasing 1% at 140 atmospheres. Sealing of the cuvette chamber was obtained by a Neoprene "O" ring squeezed between the windows. Entrance to the cell was by means of a number 20 hypodermic needle which pierced the "O" ring. Through this needle blood could be introduced by a number 25 needle. The cell could be cleaned by rinsing it with 0.17 *M* saline solution, which was then removed by suction. A stainless steel ball was kept in the cuvette for stirring. The cuvette was dropped into an insulated water bath occupying the position where ordinarily the cuvette holder is placed on the instrument. Water of constant temperature (4° or 10° ± 0.1° C.) circulated through the water bath. Dewing of the water bath windows was prevented by keeping them wet with soap solution. To accomplish equilibration the cuvette was only partially filled with blood (see Fig. 1), and it was rotated back and forth through an arc of 45° by means of a motor. To permit rotation the high pressure line of small gauge copper tubing was wound as a loose helix around the cuvette water bath, seven to eight turns, before it was connected to the oxygen cylinder. The oxygen pressure was regulated by means of a long handle on the main oxygen valve, and the pressure was read on a conventional high pressure gauge (10–140 atm.). Between one and 10 atmospheres the pressure was read on a special low pressure gauge, which could be shut off from the main line at pressures above 10 atmospheres. The "O" ring was always kept wet when under oxygen pressure and so far has not exploded.

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[4] In the paper of Scholander and van Dam (1953) the maximum depth of *Sebastes* is given as 1680 m., as recalculated from 917 fathoms given in Goode and Bean (1895, p. 261). This, however, represents a misquotation of the original figure of 179 fathoms (Townsend, 1901, p. 398).

[5] This was kindly lent to us by the Department of Biological Chemistry, Harvard Medical School.

The extinction of the blood at various oxygen tensions was read through a Wratten red filter, number 29F, and was compared with a constant gray filter held in a dry dummy-cuvette on the other side. This red filter gives the greatest separation between the oxy- and reduced hemoglobin. A gray filter (from a series of variously exposed photographic negatives) was chosen which gave a suitable reading for the oxygenated blood. As the light path on both sides was restricted to a diameter of one quarter of an inch, no wider diaphragm openings could be used than those corresponding to 0.50 on the extinction scale. The reading from full oxygenation to full reduction usually went from around 0.50 to about 1.20–1.30, giving a resolution of this extinction interval of about 2%.

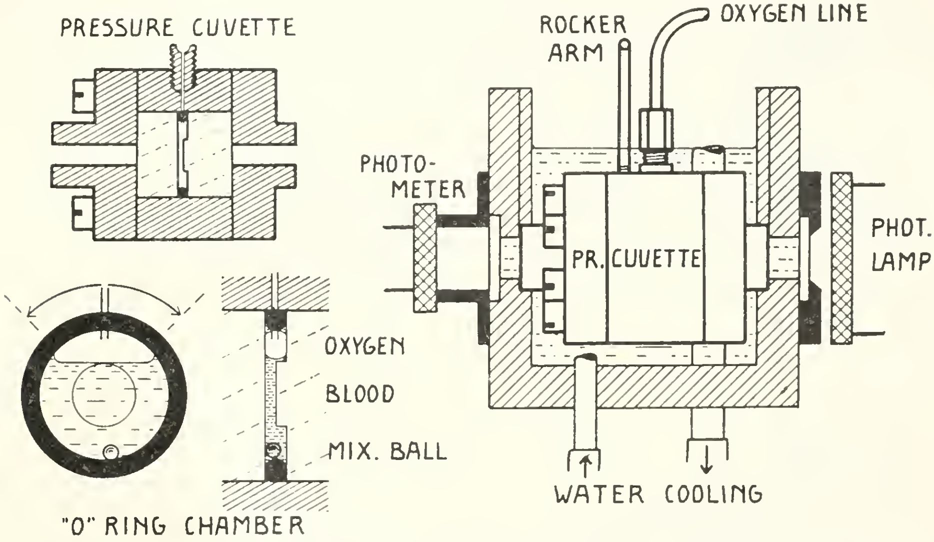

FIGURE 1. Cuvette arrangement for a study of high pressure dissociation curves.

Procedure

The heparinized blood, kept on ice, was strained, if necessary, through a few layers of gauze. Blood was transferred into three 20-cc. Erlenmeyer flasks, A, B, and C, 3.0 cc. in each. To A was added 0.3 cc. 0.17 M NaCl. To C the same amount of 0.17 M lactic acid solution was added very slowly while agitating the blood. To B was added 0.3 cc. of an equal mixture of 0.17 M NaCl and 0.17 M lactic acid. Samples A, B, and C hence contained 0, 8, and 16 millimols added lactic acid. This would drop the pH from A to B by approximately 0.7 pH units and from A to C by approximately 1.5 units. The flasks were provided with stoppers pierced with a glass tubing 6 inches long, open at both ends. They were then rotated in a water bath of the desired temperature (10° or 4° C.). A fine suction tube was passed frequently into the flask through the glass tubing to renew the air. After about 20 minutes the blood would be well aerated. Sample A was taken out and its pH taken on a Cambridge Electron Ray pH Meter. The cuvette was provided with a water jacket to keep the blood at the desired temperature. If it was below about 7.4 a small known amount of NaOH would be added very carefully until a pH of about 7.6—7.8 was obtained. The flask was rotated again and the blood was then transferred to the pressure cuvette, which meanwhile had been temperature-equilibrated in its water bath on the photometer. Rocking of the cuvette was started, and the extinction was read as soon as a constant reading could be obtained. The oxygen line was flushed and connected with the cuvette and the pressure set to give one atmosphere O_2 in the cuvette (= 1.8 atm. total).

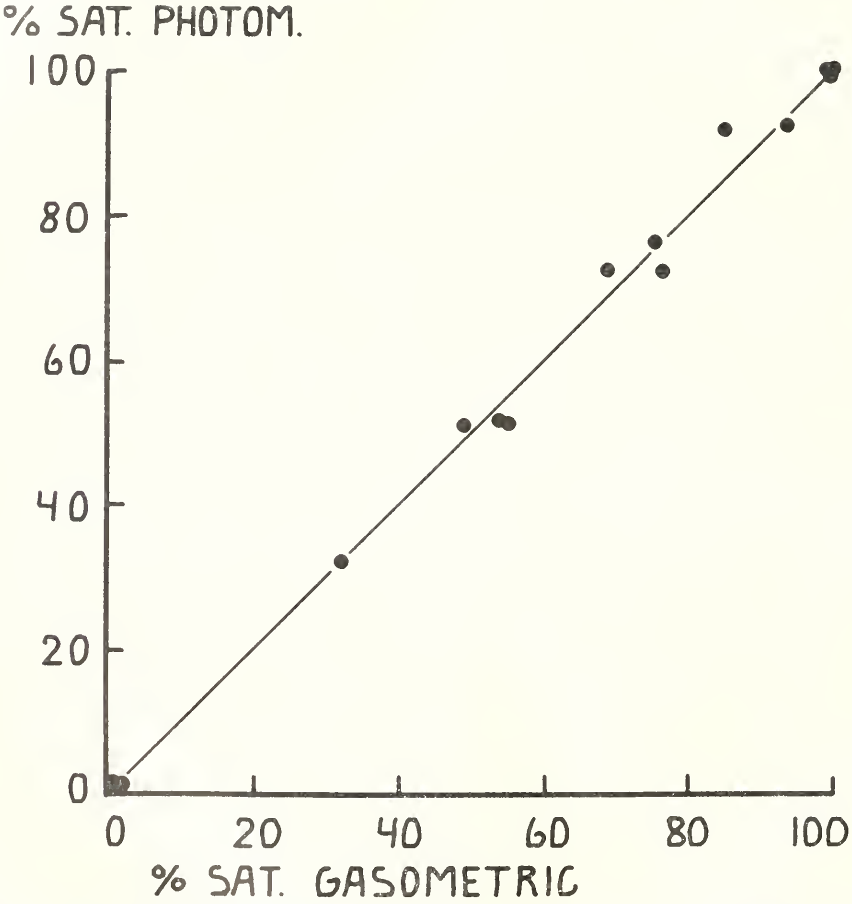

FIGURE 2. Correlation between optical and gasometric determinations of the oxygen saturation in the blood of tautog.

After about 5–10 minutes' equilibration the reading would be constant, and the pressure was raised to two atmospheres' O_2 (= 2.8 atm. total) and so on. Our oxygen contained less than 0.01% CO_2 and 0.02% N_2.

Meanwhile the remainder of sample A (about 2 cc.) was put in a rotating 50-cc. syringe, which was repeatedly flushed with helium (containing less than 0.01% O_2) for reduction of the hemoglobin. The helium was ejected and replaced four or five times, during a total equilibration time of about three quarters of an hour.

The pressure-equilibrated blood was removed from the cuvette by suction, and the cuvette thoroughly rinsed with cold 0.17 *M* saline. It was then flushed with helium and the reduced blood was introduced, completely filling the cuvette, without any contact with air. The rocker was started and the extinction of the reduced blood read.

Sample B was now taken from the equilibrator, part of it transferred to the pressure cuvette, and part of it to the pH meter. The whole procedure was then repeated for B and finally for sample C. The oxygen saturation was calculated by taking the fully saturated reading as 100% and the reduced as 0%, interpolating the intermediary readings according to a linear relation which had been found to hold for tautog blood (Fig. 2).

In cases where the effect of various CO_2 tensions was tested (at Bimini only), CO_2 and air mixtures were made up in a small spirometer and accurately analyzed (Scholander, 1947). The sample was equilibrated in a 50-cc. rotating syringe with this gas mixture, which was changed four or five times. Concentrations of 5, 10, and 20% CO_2 were used, accurate to ± 0.1%. The cuvette was flushed with the proper gas mixture before it was filled with blood.

Although optical determinations of the saturation are satisfactory enough when dealing with the same blood sample, they may not be so satisfactory when comparing samples of different pH. It is not too easy to get an accurately fixed zero point for the reduced blood. Hydrosulfite as a reductant is unsuitable to use, as the blood gets darker and darker the more hydrosulfite is added. Vacuum technique involves much shaking and handling of the delicate fish blood, and easily gives evaporation errors. We have obtained most constant results by equilibrating with helium (99.99% pure). But even so, it seems that at least sometimes a change in pH by careful titration with lactic acid will change the light transmission somewhat, due to such things as change (increase) in cell size or slight degree of hemolysis and fibrin formation. We do not believe that this source of error can have seriously distorted our results, but it is likely to be the cause of much of the spread in our data. In most cases the extinction readings of the reduced blood have matched each other within a few per cent at the different pH, and nearly always the fully oxygenated values have checked each other at different pH. The latter is the most important in the present connection.

As to the acidification of the blood we know in each case exactly the amount of acid added. The pH determinations were always made in aerated samples. A certain amount of drift and unsteadiness in the pH meter seemed unavoidable when it was used on our rolling moisture-laden ship, although the meter was kept in a dry-chamber. However, the fact that the drop in pH induced by the added known amounts of acid always came out with satisfactory regularity, as well as the fact that a colorimetric method used on our last cruise gave identical results,

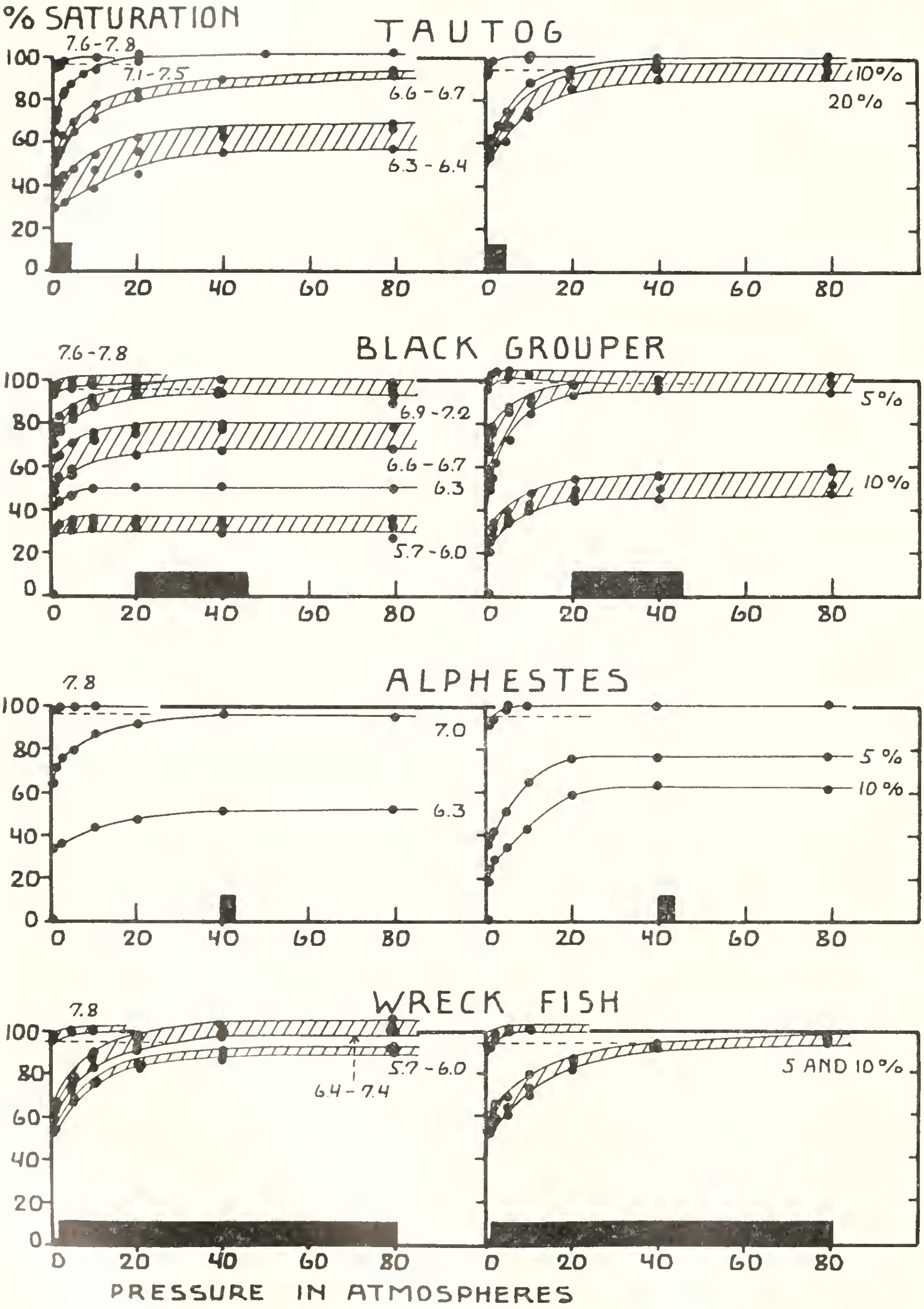

FIGURE 3. Oxygen saturation curves in various fishes at 10° C. Lactic acid was added to the blood in the curves on the left, CO_2 in those on the right. The pH values are given for curves of the lactic acid series. The equilibration pressure of CO_2 is given as per cent of one

lend assurance that our pH measurements must have been essentially correct. From very frequent checks back to our buffer standards, we consider our pH measurements to be reliable to within ± 0.1 pH unit.

RESULTS

In Figures 3 and 4 the oxygen dissociation curves are plotted for fishes living from shallow surface waters to the abysmal depths. The curves span a range of pH from 8.0 to 5.6, and the carbon dioxide tensions used were 0.03, 5, 10, and 20% of an atmosphere.

It will be seen that in half of the eight species of deep sea fishes investigated (grouper, *Alphestes,* long-finned hake, ratfish) lactic acid in a concentration of about 100–150 mg. % (pH 6.7—5.6) produces a clear Root effect, in that the hemoglobin does not become more than 30–80% saturated at tensions as high as 140 atmospheres.[6] The curves run horizontal from above 20 to 30 atmospheres. In these cases, therefore, the Root effect could be responsible for the oxygen secretion.

In the remaining four deep sea species (rosefish, wreckfish, blue hake, long-nosed eel), however, even at pH 6 the hemoglobin became 90% or more saturated when the pressure exceeded 50–100 atmospheres. The arterial saturation at 0.2 atmosphere is no more than 90–95% at pH 7.6—7.8. Since the oxygen tension in the swimbladder frequently exceeds 50–100 atmospheres it appears impossible that the Root effect can account for gas secretion in these cases, unless possibly the pH falls below 5.6—6 in the blood vessels of the gland.

It seems dubious that the pH in the blood could ever reach such a low value as 6.0. If we consider pH 6.5 as the lowest physiological limit, then only the black grouper and possibly *Alphestes* would be able to maintain part of the hemoglobin in a reduced state in the presence of the high oxygen tension. Of course we do not know what the pH might be in the capillary blood passing through the gland. The lowest pH found in the blood of dead asphyxiated fishes was 6.9. At the highest acidity used *in vitro* it was impossible to avoid the precipitation of some fibrin, and hemolysis occurred with the addition of very little more acid.

If a high CO_2 tension were to be considered responsible for the oxygen secretion via the Root effect, then the CO_2 must be present at high concentrations in the blood vessels of the gland in which case it would diffuse with the oxygen into the bladder where its concentration can be measured. In shallow fishes swimbladder CO_2 is usually below 1–2% (Table I). In the wreckfish the oxygen secretion could not be explained by the Root effect unless the CO_2 were more than 10% of an atmosphere, and the tautog would require more than 20% CO_2 to secrete in deep water. Accurate determinations were made of the CO_2 tension in the swim-

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[6] The Root effect was described from blood of shallow water fishes, dealing with oxygen pressures of no more than 700 mm. Hg (Root, 1931).

atmosphere. The broken line (90–95% saturation) denotes the arterial saturation at 0.2 atmosphere O_2 pressure. The solid bar on the base line is the range of depth in atmospheres where these species live. For each range of pH or CO_2, 3 or 4 separate runs with different bloods were usually made to get an impression of the individual variability. All curves were continued to 140 atmospheres' oxygen pressure, but showed no change from the values at 80 atmospheres.

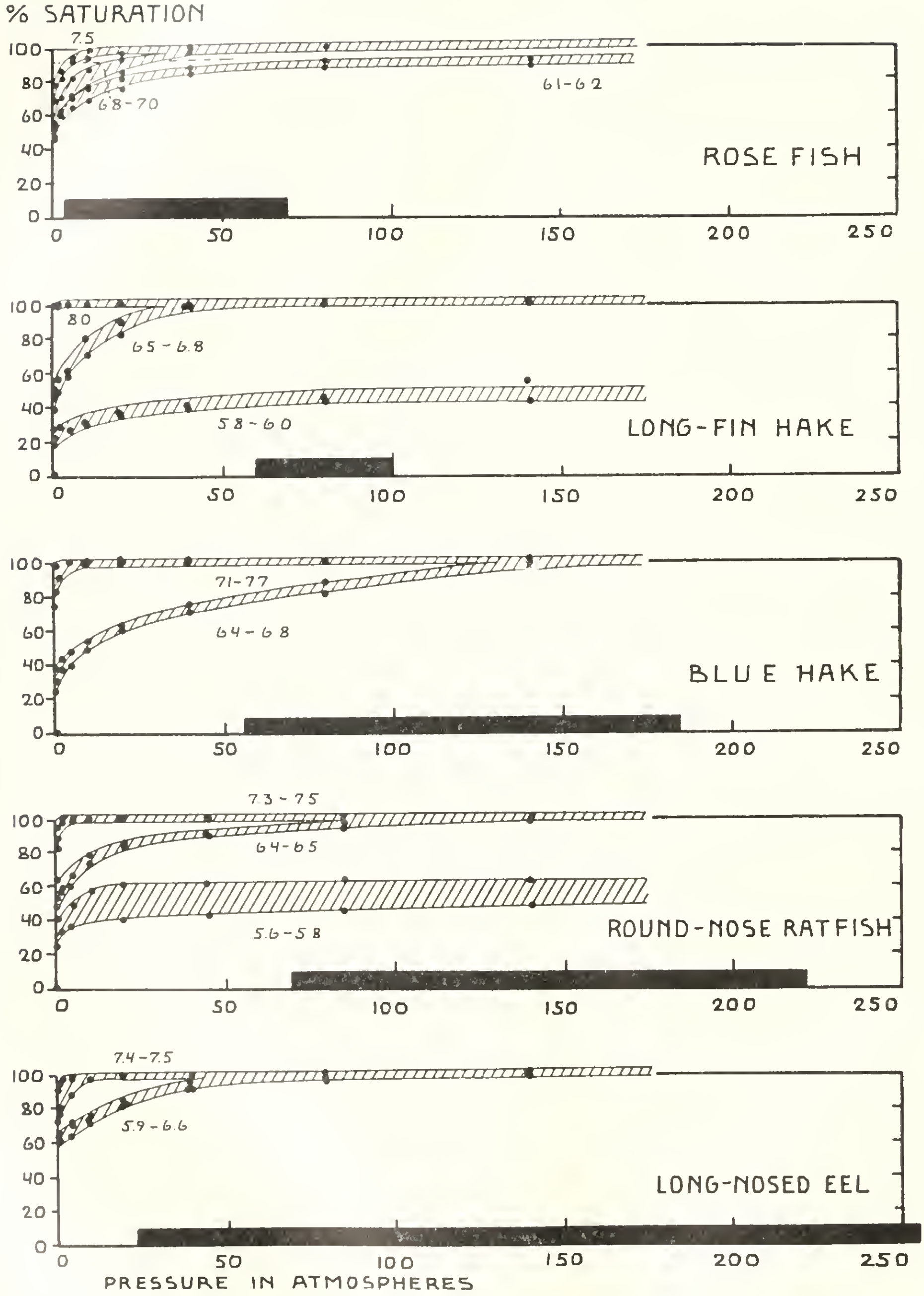

FIGURE 4. Oxygen saturation curves in blood of deep sea fishes at 4° C. The upper curve in each diagram represents the blood untreated, directly from the fish. In the other curves lactic acid has been added to give, after aeration, the pH stated for each curve. The solid bar on the base line is the depth range at which the fish lives. Each pH range was generally covered by two or three separate runs, using blood from different specimens. Arterial saturation would be no more than 90–95 vol. %.

bladder gas of the Bimini fishes (Table I, B), which were brought quickly to the surface. Assuming that only insignificant changes took place during the 4–7 minutes of getting the fish on deck, the tensions in the swimbladder gas were from 10–19% of one atmosphere CO_2 at the bottom. As the gas in the blown-up fish was dispersed all through its body cavity, it may well have picked up some CO_2 from the tissues.

If, as indicated by Root and Irving (1943), the CO_2 effect is simply a pH effect, then CO_2 cannot be the direct cause of the hypothetical oxygen dissociation from hemoglobin in our deep sea fishes. It was suggested by Black (1946) that HCl might be secreted rather than lactic acid. In tautog blood at pH 6.8 we found no greater Bohr effect with HCl than with lactic acid, and hence HCl does not seem to have any specific action beyond the pH effect.

In a discussion of the probable function of the *rete* it will be shown that in that connection, also, there are serious obstacles to regarding the Root effect as the basic chemical reaction responsible for the oxygen secretion in deep sea fishes (Scholander, 1954).

TABLE I

Carbon dioxide content of swimbladder gas

Species	Depth of catch (m.)	Number of specimens	% CO_2 Low—High	Mean partial pressure of CO_2 $\left(\frac{\text{atm.}}{100}\right)$
A. Tautog (*Tautoga onitis*)	0–1	2	0.49–0.99	0.7
Queen triggerfish (*Balistes vetula*)	0–2	7	0.33–0.99	0.4
Blue parrotfish (*Scarus caeruleus*)	0–2	1	1.02	1.0
Black durgon (*Melichtys piceus*)	0–2	1	1.08	1.1
Sergeant major (*Abudefduf saxatilis*)	0–1	5	0.29–0.48	0.4
B. Black grouper	320–400	6	0.17–0.40	10
Alphestes	408	1	0.47	19
Wreckfish	640	1	0.22	14

Mean value for partial pressures of CO_2: Group A, 0.9% of 1 atm.; Group B, 14% of 1 atm.

We wish to express our gratitude to Dr. C. M. Breder, Jr., Chairman of the Department of Fishes, American Museum of Natural History, for arranging our stay at the Lerner Marine Laboratory. We are much indebted to Mr. Michael Lerner, who helped us with advice and generously supplied us with equipment and machinery for deep sea fishing. Mr. and Mrs. Marshall B. Bishop spared no effort in facilitating our work at the Lerner Marine Laboratory. We have benefited much from advice and stimulating discussions with Dr. A. C. Redfield of the Woods Hole Oceanographic Institution, and we are deeply grateful to Mr. W. C. Schroeder at the same institution for all possible cooperation on the deep sea cruises which he conducted off the New England coast. Mr. C. Grant and Mr. J. L. Allen gave invaluable help in the construction of the optical instrumentation, and Mr. K. Morrison and Mr. Stanley N. Eldridge in building the deck laboratory.

Mrs. Susan I. Scholander has given us much valuable assistance in the laboratory and the field and with the manuscript.

SUMMARY

1. Oxygen dissociation curves at a P_{O_2} running from 0.2 to 140 atmospheres have been obtained from eight species of deep sea fishes, at a pH varying from 8.0 to 5.7 and at a CO_2 tension from 0.03 to 10–20% of one atmosphere.

2. The Root effect in acidified blood could be demonstrated in some of these species even up to 140 atmospheres, inasmuch as part of the hemoglobin remained unsaturated. In other species full arterial saturation occurred, however, at pH 6 or lower, at oxygen tensions much lower than those existing in the swimbladder. Hence the Root effect is not the mechanism for the oxygen secretion in deep sea fishes. If oxygen is derived from oxyhemoglobin it must be unloaded by some mechanism as yet unknown.

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SECRETION OF GASES AGAINST HIGH PRESSURES IN THE SWIMBLADDER OF DEEP SEA FISHES.

II. THE RETE MIRABILE[1]

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The anatomical structure in the swimbladder of physoclist fishes associated with the gas secretion is the so-called red gland. This consists of a glandular, often button-like, structure exposed to the lumen of the swimbladder. The gland is provided with blood circulation through a so-called *rete mirabile* which consists of intermingled, parallel, unbranched, afferent and efferent capillaries. In deep sea fishes these may be more than one cm. long. In the outside layers of the swimbladder these capillaries unite to form the respective arteries and veins (Fig. 1). The structure as it varies in different fishes has been describd by Woodland (1911).

The essential feature from a functional standpoint seems to be that the *rete* represents a counter current exchange mechanism where the ingoing blood is brought into intimate diffusion contact with the outflowing blood (Müller, 1840; Woodland, 1911; Haldane, 1922; Hall, 1924; Jacobs, 1930; compare Scholander and van Dam, 1954). In principle the situation can be presented as in Figure 4.

It is obvious that to maintain a gradient of 100 atmospheres or more, the millimeter-thick swimbladder wall must be very impermeable to cut down diffusion losses. This seems to be accomplished by incorporating in the wall various kinds of solids (crystals and or fibers). In the long-nosed eel the swimbladder is strikingly silvery.

At 100 atmospheres' O_2 pressure, one volume blood would dissolve four volumes oxygen. It is therefore clear that the blood flow leaving the swimbladder would cause an impossibly great loss of oxygen unless the tension in the blood were somehow reduced before it left the bladder wall.

The problem is similar to that facing a whale moving in ice water. How can it maintain body temperature, in spite of having the big flukes circulated? This is accomplished by having the fluke arteries completely surrounded by veins, so that the arterial heat is transferred to the veins and thus remains within the bulk of the body. This very general principle of heat exchange was described by Bazett *et al.* (1948) as applicable to the *venae commitantes* in man.

Let Figure 4, I represent a counter current heat exchanger where 0° water is flowing in. It is heated to 10° at the loop by some source and returns in intimate contact with the ingoing tubes. The heat exchange will then result in the establishment of a linear temperature gradient in the tubes, as pictured (Fig. 4, II). The temperature of the leaving water would depend upon the efficiency of the heat exchanger, and might be one degree. If the effluent water were run out separately, isolated from the influent water, the temperature would have been 10°. Hence a ten-fold saving of heat was accomplished by the counter current exchange mechanism. If the temperature of the source of heat is fixed, it should be noted that the loop could only approach this temperature, but could not reach or exceed it.

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The same situation would also obtain in regard to a diffusion exchange system in which instead of temperature we deal with gas tensions in a liquid.

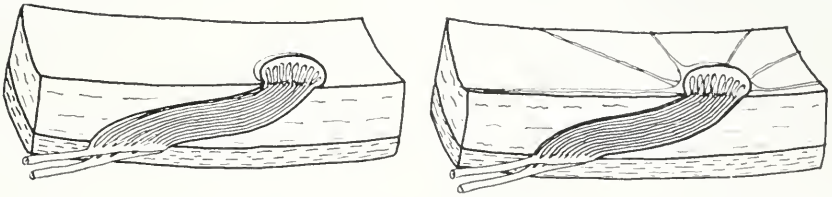

FIGURE 1. Schematic drawing of capillary *rete* and gland in relation to the swimbladder wall, which is represented with exaggerated thickness. To the left the *rete* ends in the gland (*e.g.*, in *Antimora, Coryphaenoides*) ; to the right blood vessels radiate from the gland over the surface of the swimbladder (*Sebastes, Synaphobranchus*).

MATERIAL

The deep sea material used in the present investigation was obtained from the Lerner Marine Laboratory, Bimini, and from cruises with the dragger *Cap'n Bill II* off the New England coast. (For particulars see Scholander and van Dam, 1954.)

HISTOLOGICAL STRUCTURE OF THE RETE

Sections of the *rete* from the long-nosed eel and the rosefish are given in Figure 2 (A-F). The *rete* of the rosefish is similar to that found in the common eel described by Krogh (1919). By differential injection he showed that the afferent smaller capillaries surround the larger efferent venous capillaries much in the same manner as is seen in the rosefish (D). In the ratfishes (C) these small capillaries surrounding the bigger ones are thick-walled and clearly arterial. In the long-nosed eel (A) both afferent and efferent capillaries tend to be squarish, about 13 × 13 μ. The walls between the capillaries average 1.5 μ. Most of the capillaries containing red cells are surrounded on all four sides by empty capillaries. It is believed from this that one *rete* was empty and that the *retes* are fitted together like the squares on a checkerboard.

The checkerboard arrangement is the geometrical arrangement which gives the maximal diffusion exchange between the afferent and efferent capillaries, and it is remarkable that we find it in our deepest fish. The only other solution to the topological problem of making four polygons (black or white) meet at one point in such a way that black always borders white is realized in the hexagonal star pattern found in the *rete* of the rosefish (D).

It would seem that the checkerboard pattern might have developed through a non-staggered columnar arrangement of the primary vessels (Fig. 3a), whereas the star pattern might have developed from a staggered pattern of the primary

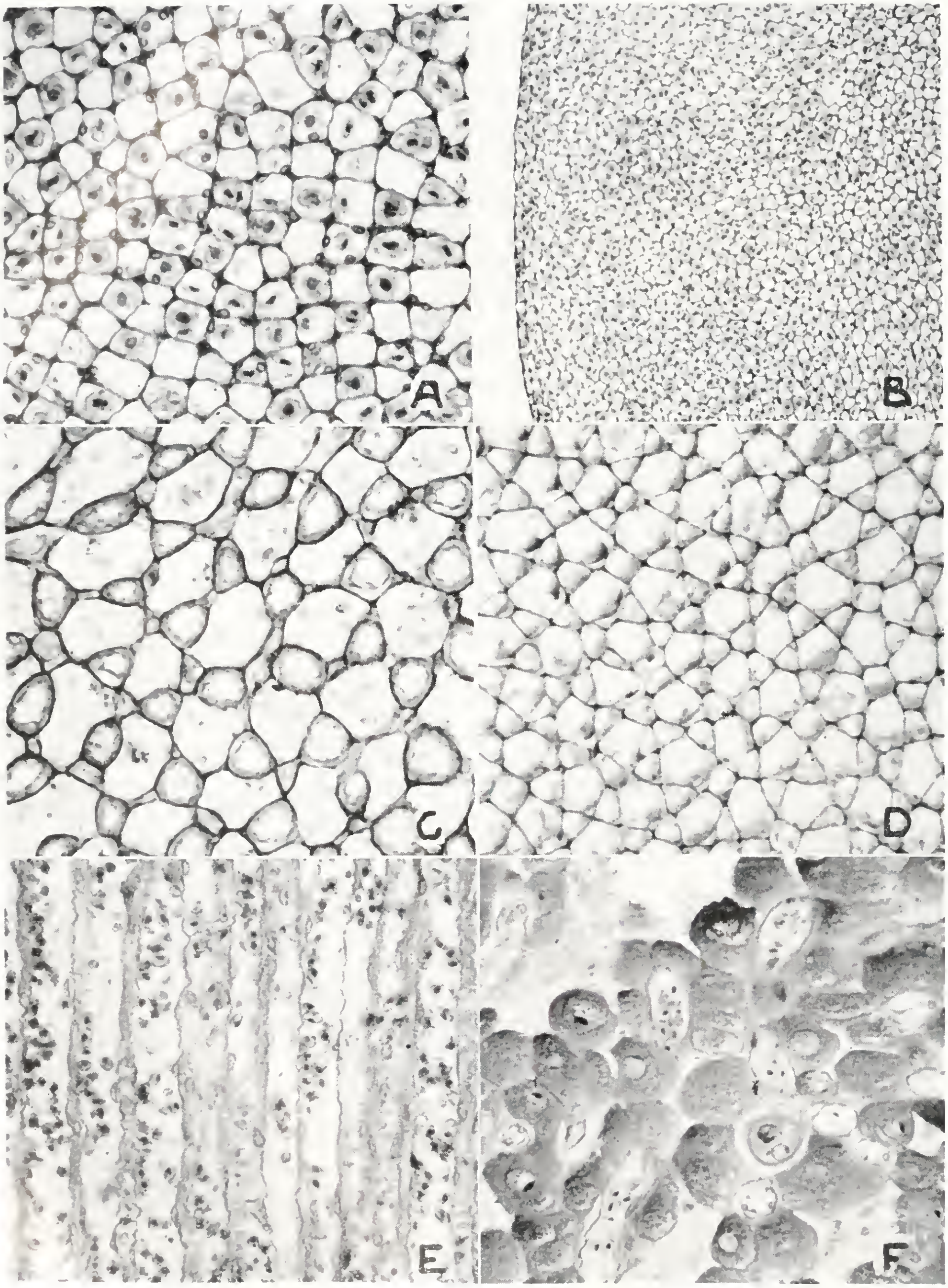

FIGURE 2. Sections through *rete* in deep sea fishes. A: *Synaphobranchus*, cross section × 300. Each polygon (square) is a single capillary. B: Same as A, × 100. C: *Coryphaenoides*, cross section × 300. D: *Sebastes*, cross section × 300. E: *Coryphaenoides*, longitudinal section × 300. Many red cells in capillaries. F: *Coryphaenoides*. Glandular epithelium from

vessels (Fig. 3b). In both cases the arterial capillaries may have developed in the interstices between the veins.

In many *retes* the loops of the glandular buttons arise from a vascular plexus located at the base of the buttons. This must naturally be so in all cases where the arterial *rete* capillaries outnumber the venous capillaries, as in the common eel, or in the rose- and ratfishes.

The length of the *rete* capillaries varies somewhat with the species, and increases generally with the depth. Measurements are given in Table I.

Figure 2F shows the surface layers of the gland "button," with capillaries surrounded by the thick glandular epithelium.

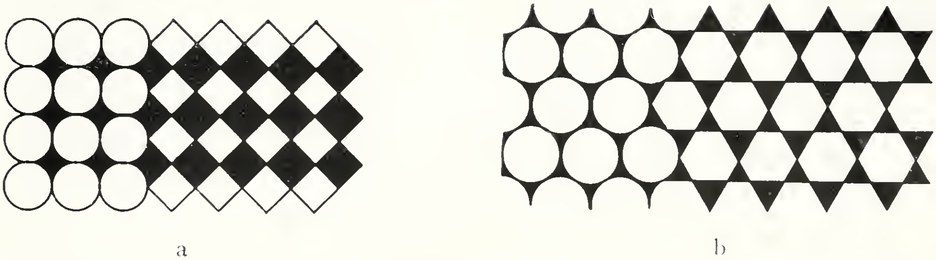

FIGURE 3. a. Possible development of the checkerboard pattern of the *rete* in *Synaphobranchus,* the primary capillaries (veins) in non-staggered rows with the arterial capillaries in the interstices. b. Possible development of *rete* in rosefish, the primary capillaries (venous) in staggered rows with the arterial capillaries in the interstices, resulting in the hexagonal star pattern.

DIFFUSION CHARACTERISTICS

From the structure and dimensions of a *rete* such as in the long-nosed eel, we may estimate what diffusion characteristics a given *rete* may have.

Let us take as unit a rete 1 cm.^{2} in cross section and 1 cm. long with capillary characteristics of the deep sea eel. The capillaries average 13 μ each side, making the circumference 52 μ. Length is 1 cm. and hence the surface area of each capillary tube is 0.0052 cm.^{2} Due to the checkered arrangement all of this surface is available for diffusion. The wall between two capillaries averages 1.5 μ. There are about 500,000 capillaries per cm.^{2} Using a diffusion constant of 150 mm.^{3} μ/cm.^{2}/min./atm. (Krogh, 1919, p. 195), we find that the diffusion across the length of this one capillary is then 0.52 mm.^{3}/min. atm. per capillary. There are 250,000 pairs of capillaries in this *rete*, and hence the total diffusion, Q, is 130 cc./min./atm.

SYMBOLS AND BASIC EQUATIONS

It is of interest to formulate a few relations pertaining to such a counter current system in order to be able to estimate more closely what a fish *rete* might be able to do.

"button," × 225. Several capillaries are seen buried among the large glandular cells. (Staining: A, B: Gomori's chrome alum-hematoxylin and phloxine, Bouin. C: Periodic acid-Schiff, Bouin. D: Periodic acid-Schiff, 80% alcohol. E: Hematoxylin and phloxine, Bouin. F: Gomori's chrome alum-hematoxylin and eosine, Bouin.) Sections and photographs by courtesy of Dept. Anatomy, Harvard Medical School.

TABLE I

Size of fish versus length of rete mirabile

Species	Depth of catch (m.)	Length	
		Fish (cm.)	*Rete* (mm.)
Rosefish	600	46	7–9
Rosefish	600	45	8–9
Rosefish	600	32	7–8
Rosefish	600	32	7–10
Rosefish	600	32	8–10
Blue hake	900	38	7–8
Blue hake	900	37	9–10
Blue hake	900	17	9
Blue hake	900	10.5	9
Common ratfish	600	34	11–13
Common ratfish	600	28	8–9
Common ratfish	600	18	8
Common ratfish	600	16	7–8

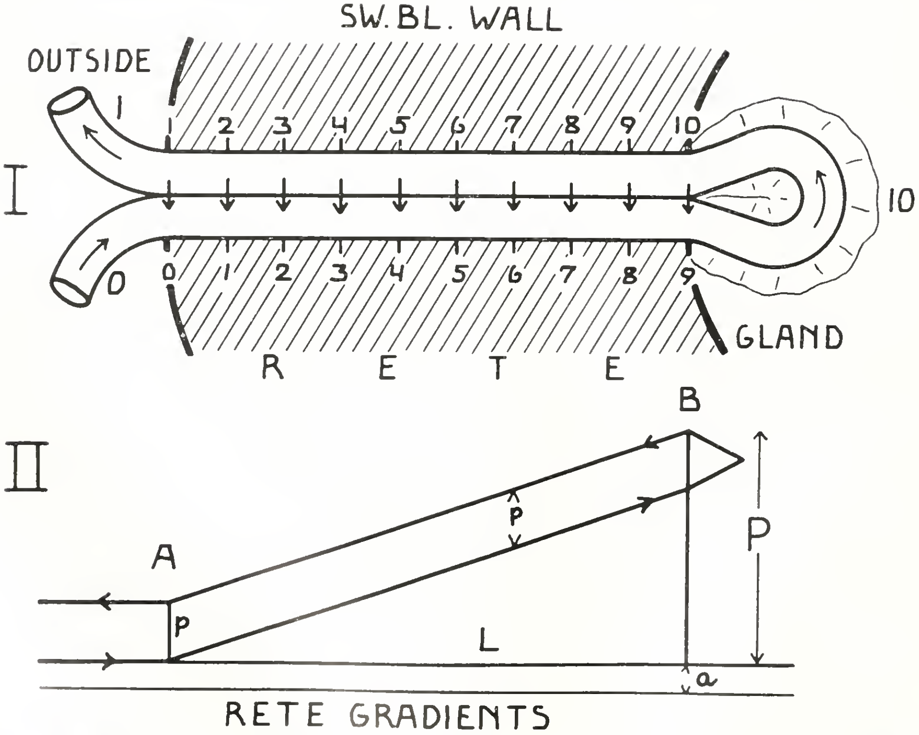

FIGURE 4. Schematic presentation of the counter current exchange principle in the swimbladder. I. Afferent and efferent capillaries. The numbers illustrate the linear gradient set up from the inside to the outside of the structure. The arrows signify the constant trans-*rete* diffusion gradient. II. Graphic representation of the gradients. L is length of the *rete;* p is trans-*rete* gradient; P is gas tension in the swimbladder; a is gas tension of inflowing blood.

Hargitay and Kuhn (1951) have developed an extensive physical treatment of this principle in relation to its probable applicability to the kidney function. Their "hairpin counter current multiplier system" is the loop of Henle. The primary concentration difference which is augmented by the system is discussed on the assumption of a semi-permeable membrane separating the afferent from the efferent flow. The difference in hydrostatic pressure due to flow presses water through the membrane and results in the primary concentration difference.

In the fish *rete* we are dealing with a different situation, inasmuch as it may be assumed that the capillaries are extremely permeable to lipid-soluble substances, especially oxygen and carbon dioxide (Pappenheimer, Renkin and Borrero, 1951), very much more so than to water. Hence it is possible to discuss the diffusion exchange in the *rete mirabile* on much simpler terms, namely, relative to what happens to gases or solutes which are simply added to the blood. The treatment is presented, realizing that it is grossly oversimplified, and at the same time it is a pleasure to credit Hargitay and Kuhn for their introduction and interpretation of the hairpin multiplier system in biology.

The following symbols will be used in the subsequent discussion:

Length of rete = L cm.
O_2 pressure in blood entering afferent *rete* = a atm.
Trans-*rete* gradient (O_2 tension gradient between afferent and efferent *rete*) = p atm.
O_2 equilibrium pressure in swimbladder = P + a atm.
O_2 pressure in blood leaving efferent *rete* (A) = p + a atm.
Blood flow = V cc./min.
Trans-*rete* diffusion = Q cc./min./atm./cm.
Solubility coefficient for oxygen in blood = α cc./cc./atm.
O_2 secreted from blood = D cc./cc.
O_2 loss by respiration and diffusion = R cc./min.

Based on the present fragmentary knowledge it seems possible that the oxygen can be secreted in two different ways: (a) molecular oxygen is directly split off from some compound in the blood, (b) oxygen in some form is transported to the glandular cells and stored there as a compound ready for intermittent use. These two possibilities will be dealt with separately.

a. *Release of oxygen directly from the blood.* It is here assumed that some constituent, at the present unidentified, is capable of dissociating off free oxygen in the blood against very high oxygen tensions. As blood cannot be stored in the gland to any significant degree, the release of oxygen stands in direct time relation to the blood flow. The fraction of the oxygen which is dissociated off and which stays dissociated off is called D and is without any connotation as to what is the nature of the responsible compound.

The diffusion characteristics of the *rete* may now be formulated in two basic equations (Fig. 4 II).

$$P\alpha V = pQ + p\alpha V \qquad (1)$$

$$DV = p\alpha V + R \qquad (2)$$

Equation 1 states that the amount of physically dissolved oxygen entering the effluent *rete* per minute (at B, Fig. 4) equals a fraction diffusing across the *rete* plus a fraction leaving the effluent *rete* (A). Equation 2 states that the amount of bound oxygen to be secreted, which enters at A, equals the amount of physically dissolved oxygen leaving at A plus an amount of oxygen lost from the swimbladder by other avenues (respiration, diffusion loss, etc.).

(i) *Quantitative evaluation of the rete function.* The oxygen secretion will first be dealt with without considering the term R. In equation 1 the term $p\alpha V$ is negligibly small compared to $P\alpha V$ and can be omitted. We then get the following relation:

$$\left.\begin{aligned} P\alpha V &= pQ \\ DV &= p\alpha V \end{aligned}\right\} \qquad (3)$$

and therefore

$$P = \frac{DQ}{\alpha^2 V} \qquad (4)$$

The equilibrium pressure P is consequently inversely proportional to the blood flow and directly proportional to the amount (D) of dissociated oxygen per cc. blood. P is also proportional to Q and hence to the length of the *rete*. This is borne out by the observation that for a given depth and for a given species both small and large individuals had the same length of *rete*, although the cross sections varied markedly with the size (Table I). One might make an analogy between length of *rete* and voltage, between cross section of *rete* and current (amps.).

We shall consider the *rete* equations in relation to a hypothetical but plausible deep sea fish with the following constants:

Weight	1000 g.
Swimbladder volume	50 cc.
Oxygen consumption, 8° C.	1 cc./min.
Respiratory need of swimbladder and other losses = R	0.01 cc./min.
Oxygen secreted from blood = D	0.05 cc./cc.
Oxygen solubility coefficient = α, 8° C.	0.04 cc./cc.
Trans-*rete* diffusion = Q	130 cc./min./atm.

Q varies greatly from species to species and can be given great latitude.

Let us assume a blood flow (V) through the *rete* capillaries of 1 cc. per minute and that 0.05 cc. O_2 per cc. blood (= D) is dissociated off by action of the gland. The oxygen secretion would then amount to only 50 mm.^{3}/min. or 5% of the total oxygen consumption. According to equation 4 the equilibrium pressure would then be no less than 4060 atmospheres. Obviously, therefore, if there are no losses and if the dissociation is not pressure-sensitive, the potential pressures are enormous, and would increase with lowering of the blood flow.

It is necessary to introduce a term (R) expressing the loss of oxygen by such factors as the respiratory need of the gland and the lining of the swimbladder, diffusion loss, and oxygen used for compression work.

The equations would then be:

$$\left.\begin{aligned} P &= p\frac{Q}{\alpha V} \\ DV &= p\alpha V + R \end{aligned}\right\}$$

or

$$P = \frac{(DV - R)Q}{\alpha^2 V^2} \tag{5}$$

It is expedient at this point to consider what rates of oxygen deposition might be pertinent.

Bohr (1905) found that cod could fill its swimbladder with oxygen in about one day. Akita (1936) found the same rate for *Monacanthus.* Copeland (1952) found that *Fundulus* would need about two days, if it were able to maintain full rate for so long. If the cod and *Monacanthus* were to fill their bladders at 100 atmospheres' pressure it would take 100 days to do it. Such a drastic thing is hardly relevant for a deep sea fish. It may, however, have to compensate for a buoyancy change of, let us say, 10%, by moving from 900 meters' depth down to 1000 meters. This would require 10 days of secretion. The quantitative development of the *rete* varies greatly from species to species. Suppose that our fish can do twice as well as the cod. It would then need five days to accomplish the 10% buoyancy change and would secrete at a rate of 100 cc. oxygen of one atmosphere a day, or approximately 70 mm.^{3}/min. This would represent 7% of the total metabolic rate of the fish. Let us assume that the respiratory and diffusion loss (R) adds to it 10 mm.^{3}/min.[2] This would then give a total release of 80 mm.^{3}/min., which would require that 1.6 cc. blood per minute enter the *rete.* The potential pressure developed using these figures in equation 5 would come out as 2220 atmospheres.

When the necessary amount of gas has been deposited to accomplish the buoyancy change the fish must shut down the deposition to a simple maintenance level. This may very possibly go via a reduction of R, which may drop to, let us say, 5 mm.^{3}/min. This, according to equation 5, would make the blood flow (V) very slightly more than 0.1 cc./min., *i.e.,* 16 times less than at full secretion rate. During activity the blood vessels of the gland dilate markedly (Hall, 1924; Fänge, 1953). A less economical way of maintaining constant swimbladder volume at 100 atmospheres (or of reducing the volume) would be to draw some blood off through the non-*rete* system, like the capillaries of the "oval." Then 67 mm.^{3}/min. of O_2 would have to be run off from the swimbladder, which would require that 4 mm.^{3} blood per minute be bled through the oval.

(ii) *Amount of oxygen secreted per cc. blood at steady state.* By considering the maximal rate at which oxygen can be deposited in the swimbladder one may calculate D, *i.e.,* the amount of dissociable oxygen carried in by the blood.

If, again, 80 mm.^{3}/min. is taken as the maximum rate of secretion and if the blood flow (V) through the *rete* is 2 cc./mm., the blood would have to unload 40 mm.[3] oxygen per cc. blood, or more or less what the hemoglobin could do if dissociated.

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[2] The work of compressing one mol gas from 0.2 to 100 atmospheres at 8° is equal to $1.99 \times T \times 2.3 \log (P_1/P_2)$ Cals. = 3.5 Cals. The caloric equivalent of one liter O_2 equals 4.8 Cal. or 104 Cal. per mol. Hence for each part of oxygen secreted into the swimbladder a minimum of 3% goes to the work of compression, or 2.1 mm.^{3} out of the above 10 mm.^{3} which constitute R.

The amount of physically dissolved oxygen in the blood is no more than 0.04 × 0.2 = 0.008 vol. % and therefore cannot be the source of a steady-state secretion, even if the solubility coefficient in the effluent blood were drastically lowered by some unknown process. The tension difference (p) between affluent and effluent blood could only be a few millimeters Hg.

b. *Secretion of oxygen from the gland cells.* Considering that we have failed to identify hemoglobin or any other compound in the blood as the direct source for the secreted oxygen, we must consider the possibility of a cellular secretion, where the oxygen is ultimately transported to the glandular cells via some compound in the blood. This compound, or a derivative thereof, might be stored in the epithelial cells for intermittent use. Non-steady-state events are conspicuously associated with the secretion. Glycogen is thus stored in large quantities by the gland cells if secretion is inhibited. When the gland is secreting, the glycogen is being used, and secretion cannot go on when the store of glycogen is depleted (Copeland, 1952; Fänge, 1953). If a breakdown of a stored compound is what determines the maximal rate of oxygen secretion, then the amount (D) of oxygen transported to the gland per cc. blood can be much smaller than would be necessary if the oxygen is released directly from the blood. From the information available it is impossible to decide between cellular secretion of oxygen and the release of molecular oxygen directly from the blood.

Conclusions. The discussion of the *rete* may be summarized in the following statements. The structure of the *rete* is such as to provide for an extremely efficient counter current diffusion exchange mechanism between inflowing and outflowing blood. This makes possible a steep tension gradient within the *rete* so that tension loss through the circulation becomes very low. Quantitative considerations of the diffusion efficiency of the *rete* make it clear that the limiting equilibrium pressure theoretically could reach tremendous values, provided the dissociation pressure of the underlying chemical or physical reaction is even higher. If oxygen cannot be chemically stored in the gland it must enter through the blood concomitantly with the secretion. This would require an amount of oxygen per cc. blood larger than that physically dissolved in the arterial blood. The nature of the responsible compound is not known.

THE ROLE OF LACTIC ACID AND CO_2

We have seen that thanks to the multiplication principle of the *rete,* small primary concentration effects can build up to considerable pressures, limited ultimately by the pressure characteristics of the primary chemical or physical dissociation. It was shown that the Root effect or Bohr effect at pH 6 was nullified already at 50 atmospheres in some fishes which live and secrete oxygen at much greater depths, and therefore it could not be the primary pressure reaction. Whether stronger lactic acid and lower pH could do it we do not know. It seems difficult *in vitro* to go much further without irreversible damage to the blood (Scholander and van Dam, 1954).

As regards CO_2 it was likewise shown that in some fishes 5 and 10% CO_2 would be inadequate to dissociate off oxygen at the required pressures. The question that arises is: Could the CO_2 tension be higher than this, and if so what would be the pressure characteristics of the Bohr or Root effect?

The CO_2 content of the aerated blood of several deep sea species is given in Table II. If a small amount of lactic acid were added to the blood by the gland the CO_2 pressure would build up in the *rete* and the swimbladder, and the approximate tension gradients can be calculated, inasmuch as the diffusion constant can be taken as about 25 times that of oxygen (Krogh, 1919), and the solubility coefficient is about 1.0, or again 25 times that of oxygen. This would make $\underset{\sim}{Q}$ of equation 1 for $CO_2 = 25 \times 130 = 3250$ cc./min./atm.

If the CO_2 effect were responsible for the oxygen secretion in the wreckfish we would expect to find in the swimbladder at all times of steady-state a CO_2 tension higher than 10% of an atmosphere. A wreckfish caught at 640 meters had 0.22% CO_2 or 14% of an atmosphere. Six black groupers had from 6.6—13% of an atmosphere CO_2. In contrast to this 27 specimens of surface fishes, comprising 6 species, had a CO_2 tension averaging only 0.7%. It has been shown by Jacobs (1934) and von Ledebur (1937) in 10 species of physoclist fishes, which were induced to secretion by puncture, that on the average the CO_2 tension in the swimbladder rose by only 1.4—3.1% while the O_2 rose to 80–90%. In some of the species the CO_2 did not rise at all. A *Serranus cabrilla* with the oval obliterated accumulated no more than 3% CO_2 during the process of secretion. The relatively high CO_2 tensions in deep sea fishes may hence very likely be artifacts, as CO_2 diffuses so fast. If, nevertheless, we assume that our deep sea fish were to require as much as 0.5 atm. CO_2 in the swimbladder[3] to dissociate off the oxygen, and if we assume this Root effect to be reversible when the CO_2 pressure decreases, then we can estimate the rate at which such deposition could take place.

TABLE II

*Carbon dioxide content of blood**

Species	Depth of catch (m.)	Number of specimens	CO_2 (vol. %)	
			Range	Mean
A. Undiluted whole blood, aerated at 10° C.				
Black grouper	333–375	4	4.8–6.3	5.5
Alphestes	410	1	—	7.0
Wreckfish	640	2	6.8–8.0	7.4
B. Whole blood plus lactic acid 0.17 *M* (10:1), aerated at 10° C.				
Black grouper	333–375	2	1.4–1.5	1.45
Alphestes	410	1		1.9

* Method of Scholander, Flemister, and Irving (1947), modified.

If P = 0.5 atm., $\underset{\sim}{Q}$ = 3250 cc./min., $\alpha\, CO_2$ is 1.0, and at a high rate of secretion V = 1 cc./min., then according to equation 3

$$p = 0.12 \text{ mm. Hg}$$

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[3] Tautog blood begins to hemolyze at one atmosphere CO_2.

In other words the P_{CO_2} of the "venous" blood leaving the *rete* would be only 0.12 mm. Hg higher than that of the "arterial" blood entering the *rete*. Now the oxygen *tension* of the leaving blood is always slightly higher than the tension of the entering blood. The leaving hemoglobin would hence be nearly, if not completely, arterialized. If under these circumstances any oxygen at all were deposited it would be at an infinitesimally small rate.

If the leaving CO_2 tension were to be high enough to effect a half-saturation of the hemoglobin (which is necessary for the secretion rate), one would expect at least around 10 mm. Hg CO_2 tension in the leaving blood. This would, however, require some 43 atmospheres' CO_2 tension in the swimbladder, which of course is absurd.

We see therefore that unless CO_2 at tensions higher than we tested produces some *irreversible* oxygen-splitting from the hemoglobin, we cannot accept the CO_2 effect as producing the oxygen secretion.

Koch (1934) considered that if the elevated CO_2 tension passed onto the arterial side of the *rete*, the O_2 would increase there and O_2 would diffuse across to the venous side, which would seem like a short circuit with regard to the O_2. In order to avoid this he thought that buffer might diffuse across from the arterial side to the venous, due to hydrostatic forces. If this were to happen it would at best mean a lower P (CO_2) on the venous side, hence an even earlier recombination of oxygen with the hemoglobin before the blood left the *rete*, and hence no secretion.

According to either of the two theories (CO_2 and lactic acid) the oxygen is liberated directly from the blood and hence *in* the *rete*. This obviously wastes part of the *rete* as a diffusion barrier, and the oxygen must also diffuse from the *rete* into the swimbladder. Here we encounter another seemingly backwards situation, inasmuch as in many fishes the capillary loops in the gland are covered with a deep layer of epithelial cells (Fig. 2F), which must hinder very substantially the gas diffusion into the swimbladder. The loops are also usually very much fewer than the *rete* capillaries. In order to get an efficient gas exchange we would expect rather a great many almost naked capillaries, such as in the lungs.

The anatomical arrangement seems to fit a cellular gas secretion much better. This would leave the whole *rete* as a simple counter current diffusion barrier against oxygen loss, and the secreting cells covering the capillary loops would aid the *rete* as a diffusion barrier, rather than counteract it.

There are grave difficulties in seeing the Bohr or Root effects as the cause of the secretion, and at the present time it seems indicated that many observed events associated with the secretion of oxygen, such as the occasional large rise in CO_2 (Jacobs, 1930; von Ledebur, 1937; Rostorfer, 1942), the acid formation (Hall, 1924), the glycogen disappearance (Copeland, 1952; Fänge, 1953), carbonic anhydrase activity (Leiner, 1938, 1940; Copeland, 1952; Fänge, 1953), potential glycolytic activity, lactic acid formation (Strittmatter, Ball and Cooper, 1952; Fänge, 1953) must be fitted into the secretion picture in some other way.

If dissociation of oxyhemoglobin still is to be considered as the direct cause of the pressure build-up, it must be brought about by a diffusible x-substance (from the gland cells), which would dissociate off oxygen against much higher pressure than does lactic acid. This x-substance would either have to leave with the blood and must then be quite indiffusible through the *rete* capillaries, in contrast to the gland capillaries, or if easily diffusible it would have to effect an irreversible reaction, such as methemoglobin formation, to be repaired outside the gland. Such a substance and such diffusion conditions are purely hypothetical, although perhaps possible.

There are at the present time unresolved obstacles in regarding the Root or Bohr effect as the secretion mechanism. It has not been proved that hemoglobin is involved, and it would be premature to disregard the possibility of a cellular oxygen secretion.

Conclusions. In several deep sea fishes the blood acidified with lactic acid to pH 6 or lower arterializes fully at a much lower oxygen pressure than that existing in the swimbladder. Hence lactic acid via the Root effect cannot be the cause of oxygen secretion. If the CO_2 effect were to be the cause of the secretion, CO_2 would have to leave the gland at a pressure of at least 5–10 mm. Hg. This would require a CO_2 pressure in the swimbladder of 20–40 atmospheres, which is out of the question. If hemoglobin is directly involved in the oxygen secretion the mechanism by which it is dissociated is unknown. One is led to consider seriously the possibility of a cellular oxygen secretion.

TABLE III

Nitrogen content of the swimbladder gas in deep sea fishes

Species	Depth of catch (m.)	Number of specimens	% N_2	Mean partial pressure of N_2 (atm.)
Black grouper	320–400	6	12.5–16.8	5.4
Alphestes	410	1	18.5	7.6
Wreckfish	640	1	11.0	7.0
Rosefish	660	2	20.0	13.2
Long-finned hake	1300	5	3.0–3.7	4.4
Blue Hake	1240	4	6.0–7.6	8.0
Round-nosed ratfish	1100	3	9.4–10.0	10.8
	1200–1240	3	8.4–10.3	11.6
Common ratfish (*Macrourus bairdii*)	1240	4	7.2–7.8	9.3
Long-nosed eel	1200	1	7.6	9.1

SECRETION OF INERT GASES

It has been shown that the nitrogen tension found in the swimbladder of marine fishes is practically always higher than in the sea water. In each species the nitrogen is deposited as a relatively constant proportion of the oxygen, but the fraction varies from species to species. It usually lies between 2 and 15%. In deep sea fishes the N_2 tension can therefore reach as much as 15–20 atmospheres. New data from greater depths confirm these conclusions, Table III and Figure 5. It does not seem possible to explain the high nitrogen as an artifact (Scholander and van Dam, 1953).

The whitefish (Hüfner, 1892) and several more species of freshwater physo-

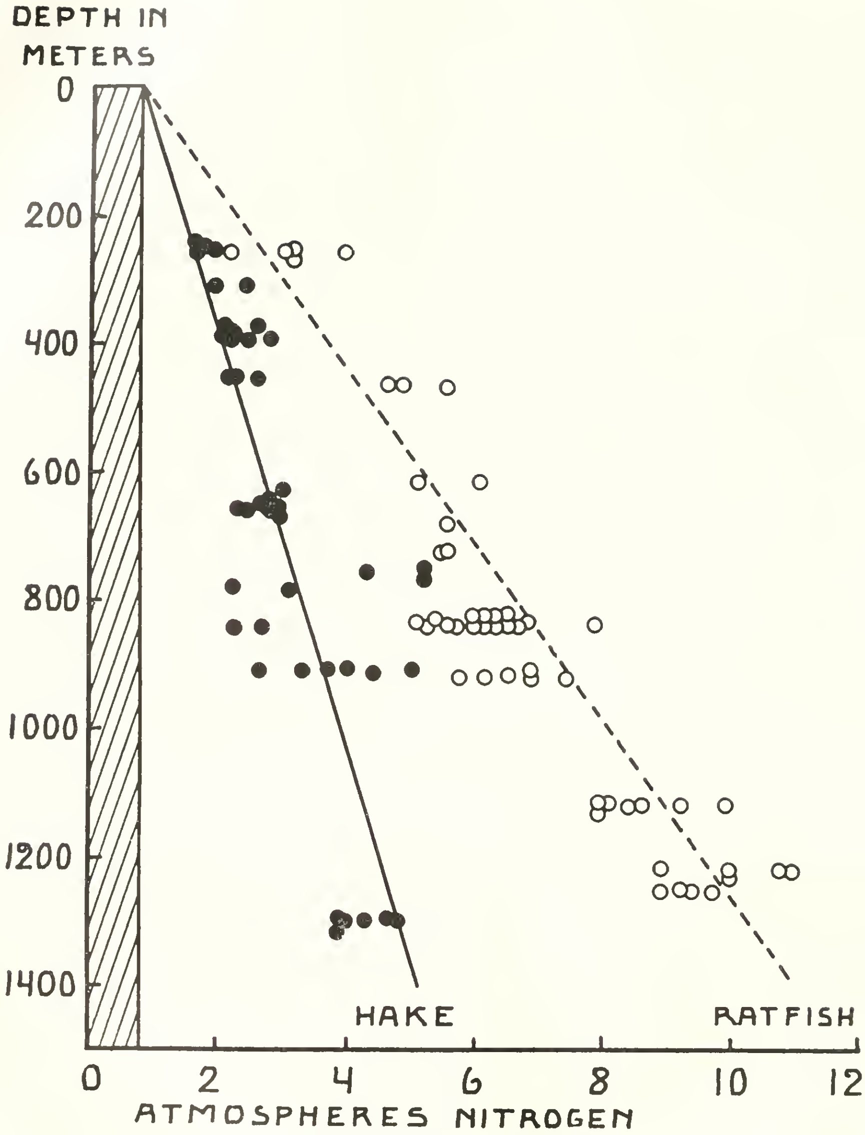

FIGURE 5. Relation of nitrogen tension in swimbladder to depth for the long-finned hake (*Urophycis chesteri*) and common ratfish (*Macrourus bairdii*).

stomes (Saunders, 1953) are able to deposit "pure" nitrogen against some 6–8 atm. pressure.

The argon-nitrogen ratio in the swimbladder. An important clue as to how basically nitrogen enters the swimbladder can be obtained by comparing the argon-nitrogen ratio in the swimbladder gas to the ratio in the air, as pointed out by Koch (1934). He considered, and rightly so, that the fact that the A/N_2 ratio found by

Schloesing and Richard (1896) was near the atmospheric ratio indicated that the nitrogen could not have been chemically secreted.

Some of our deep sea samples have been analyzed for argon. This was made possible through the courtesy of Dr. A. O. C. Nier and of Mr. Bailey Donnally, who kindly made the analyses for us on the mass spectrometer. The results will be seen in Table IV and are in favor of a physical deposition of nitrogen. If nitrogen were secreted chemically, the argon/N_2 ratio would be decreased in the given fishes between 6 and 20 times, *i.e.*, it would have been found to be between 0.2 and 0.06, instead of 1.18. Although there are substantial deviations from the air ratio they are certainly not of the order which would suggest nitrogen secretion by chemical means. Considering the fact that the argon may be expected to have twice the diffusion coefficient of nitrogen, one would expect the argon fraction to tend to be low rather than high (Scholander and van Dam, 1953).

Table IV

Argon-nitrogen ratio in the swimbladder gas of deep sea fishes

Species	Depth of catch (atm.)	% (N_2 + A)	N_2 + A atm.	$\frac{100A}{N_2}$
Air	1	80	0.8	1.18
Blue hake		6.2		1.16
Blue hake		6.0		1.22
Common ratfish	80	7.2	5.8	1.00
Common ratfish	80	7.6	6.1	1.61
Common ratfish	80	7.4	5.9	1.08
Rosefish	50	20.0	10.0	0.94
Rosefish	50	20.0	10.0	0.87
Long-nosed eel*	167			1.94
Moray eel*	9			1.85
Air*	1			1.18

* Schloesing and Richard, 1896. Long-nosed eel, *Synaphobranchus pinnatus*; Moray eel, *Muraena helena*.

There are unfortunately no data available on the argon/N_2 ratio in the whitefish, nor on the presence of organic gases, which would appear as nitrogen.

Theories for Secretion of Inert Gases

Two theories have been offered to explain the high nitrogen tensions.

a. *The bubble theory.* Powers *et al.* (1932) assumed that the Root effect would result in the formation of bubbles and that these when entering the swimbladder would transport nitrogen in from the surroundings. If the Root effect were responsible, the highest possible local pressure increase would be in the blood, and the bubbles would form there, and as Koch (1934) pointed out, it is hard to see how they would get out of the blood. Haldane (1922) pointed out that "bends" in human divers occur only if the pressure is diminished by more than 50% at a time. The highest increase in the plasma which dissociation of 9 vol. % oxygen could give is 3 atmospheres. Operating with blood equilibrated at 100 atmospheres' oxygen pressure in our cuvette, bubbles never formed at sudden drops of pressure, even ten times 3 atmospheres. Hence in deep sea fishes gas bubbles cannot very well form in the capillaries of the gland or of the *rete*. Experimentally, gas bubbles have not been observed to form within living cells, even after decompression from very high gas pressures (Harvey *et al.*, 1944). In shallow fishes, however, gas bubbles were seen to form in the mucus covering the gland when the gland was stimulated to activity (Fänge, 1953).

b. *Secretion by change in the solubility coefficient.* The second theory, proposed by Koch (1934), seems to offer a possible mechanism for the increase in nitrogen and inert gases. He considered the possibility that by some means the blood in the swimbladder vessels would have a lower solubility coefficient for gases than it does outside the swimbladder. This could come about by an increase in the temperature of the active gland, or by added solutes.

Any metabolic heat gradient would certainly be very small, as the swimbladder wall has no aspect of being a thermal insulator. Due to the extraordinry exchange capacity of the *rete* there would be, if any, only an infinitesimal difference in the temperature of blood entering and leaving the *rete*. Hence they would have the same nitrogen capacity and N_2 deposition could then not occur (equation 6).

If the solubility were depressed by the addition of some solute entering the blood from the gland, the nitrogen tension would slightly increase on the venous side. If this situation persisted so that the blood would leave the *rete* with a decreased solubility coefficient the following relation would obtain, using the symbols given above and essentially equations 1 and 2:

$$\left.\begin{aligned} P\alpha_2 V &= pQ \\ a\alpha_1 V &= (a + p)\alpha_2 V \end{aligned}\right\} \tag{6}$$

$$P = \frac{a(\alpha_1 - \alpha_2)Q}{\alpha_2^2 V} \tag{7}$$

The numerical values for the constants are: $a = 0.8$ atm.; $\alpha_1 = 0.0202$ cc./cc./atm.; $\alpha = 0.0200$ cc./cc./atm.; $V = 1$ cc./min. Q is directly proportional to the diffusion constant which for N_2 is about half that of oxygen. Q is therefore for nitrogen 65 cc./cm./cm.^{2}/min./atm.

If we have a 1% decrease in solubility at the exit of the *rete* the equilibrium pressure P would be 26 atmospheres. Theoretically, therefore, one could get enough pressure built up. It is obvious that the rate of N_2 deposition by this means would be exceedingly slow, since each cubic centimeter of blood would only transport in 0.16 mm.^{3} N_2/min.

A striking thing about the nitrogen tension in the swimbladders of our marine fishes is that in most of them it increases linearly with the depth, as if the nitrogen were produced as a constant percentage of the oxygen secretion. This observed phenomenon ($P_{N_2}/P_{O_2} = 2$–15%, and independent of pressure) could only be derived from the equations 5 and 7 if R were either proportional to the pressure or if it were constant, neither of which assumption seems likely to be true. There is a further difficulty with the proposed theory where an x-substance produces the lowering of the solubility coefficient, in that if the substance were diffusible it would be likely to concentrate enormously in the inner part of the *rete*. And if it were not diffusible how would it have entered the blood stream in the first place? Another serious difficulty is that physostomes like the whitefish evidently lack a typical *rete*.

Conclusions. The nitrogen-to-argon ratio in the swimbladder of deep sea fishes was found to be approximately that of the sea water and atmosphere, confirming what was found earlier by Schloesing and Richard (1896). In these fishes, therefore, the nitrogen has not been chemically secreted.

The theory of Koch (1934) that nitrogen and argon might be secreted into the swimbladder by a decreased solubility coefficient in the effluent blood of the *rete* has been evaluated quantitatively. Although high enough equilibration pressures of nitrogen could be achieved by a small change in the solubility coefficient, the constant ratio N_2/O_2 at all depths, as actually found, cannot readily be explained by this theory. The theory may not apply to physostomes, like the whitefish, which seem to lack a typical *rete*.

During this work I have received many suggestions from stimulating discussions with Dr. A. C. Redfield and Dr. L. van Dam at the Woods Hole Oceanographic Institution. I am much obliged to Dr. A. O. C. Nier and to Mr. Bailey Donnally at the Physics Department of the University of Minnesota for mass spectrographic analyses of the argon/nitrogen ratio in the swimbladder gases. Dr. George Wislocki, Department of Anatomy, Harvard Medical School, has generously given his help and advice with regard to the histological material. This long-preserved material was most skillfully prepared by Mrs. Edith Herman and photographed by Mr. Leo Talbert of the same department. I am much indebted to Dr. C. M. Breder, Jr., of the American Museum of Natural History, for arranging our work at the Lerner Marine Laboratory, and I wish to thank Mr. Michael Lerner and the staff of the Lerner Marine Laboratory for all the help they extended to us. Mr. W. C. Schroeder of the Woods Hole Oceanographic Institution gave us the best of facilities on board the dragger *Cap'n Bill II*, and for much assistance in the laboratory and with the manuscript thanks are due to Dr. L. van Dam and Mrs. Susan I. Scholander.

SUMMARY

1. The structure and dimensions are given for the *rete mirabile*. It is interpreted as a counter current diffusion exchange mechanism between the afferent and efferent *rete*.

2. This diffusion exchange has been quantitatively evaluated for O_2, CO_2, and N_2. The arrangement makes possible the maintenance of a steep tension gradient within the *rete*, so that the oxygen loss from the leaving blood can be extremely low.

3. Quantitative evaluation of the efficiency of the *rete* diffusion makes it clear that the limiting equilibrium pressure in the swimbladder could be extremely high, and that the limiting factor lies mainly in the dissociation pressure of the chemical or physical reaction which ultimately splits off the oxygen.

4. The nature of the responsible compound and reaction is unknown. The anatomical arrangement of the glandular structure and the *rete* is suggestive of a

cellular secretion of oxygen rather than of a mechanism that splits off oxygen in the blood.

5. Data on the deposition of nitrogen against high pressures which were previously found in deep sea fish have been confirmed and extended. The argon-to-nitrogen ratio in the swimbladder gas suggests that the nitrogen has entered the swimbladder via some physical mechanism. The possibility that this is brought about by a lowering of the nitrogen solubility coefficient in the efferent *rete* is discussed.

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THE EFFECTS OF LIGHT ON TEMPERATURE SELECTION IN SPECKLED TROUT SALVELINUS FONTINALIS (MITCHILL)[1]

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It will be generally accepted that the locomotory behavior of any animal in its ordinary life is a reaction or response resulting from the host of stimuli both internal and external which are continually acting upon it. The physiological analysis of such resultants is so obviously complex that here, as in most other fields, investigators have felt it desirable to commence investigation in simplified situations where the number of independent variables operating simultaneously is kept as few as possible. As a consequence much has been learned about the reactions of a great variety of animals to light, when temperature, gravity, humidity, nutritional state, etc., are kept constant; of the reaction to temperature if light and the other possible variables are kept constant, and so on. The relatively enormous literature which has resulted has been admirably summarized by Fraenkel and Gunn (1940).

It is evident, however, that if a complete understanding is to be obtained of the sensori-motor mechanisms and the nervous system which integrates them, experiments will be required in which the reactions to the simultaneous application of several stimuli are studied. Initial steps in this direction have already been made in a number of instances. Even Mendelssohn (1902) in his early work with protozoa showed that the responses of the organisms to two simultaneously acting stimuli were varied and depended upon the experimental arrangements. With the proper conditions the stimuli reinforced one another. Under other conditions the effect of one or the other stimulus prevailed—to a degree which was related to the relative strengths of the two stimulating conditions.

Of the stimuli which can be combined, light and temperature are among the most easily controlled from an experimental standpoint and are possibly among the most important factors affecting organisms from moment to moment. It is well known that when organisms are free to move about in a gradient of temperature they tend to be confined to some particular relatively small range of temperature which they are then said to have "selected." (See Fraenkel and Gunn, 1940; Sullivan and Fisher, 1953; Stinson and Fisher, 1953, for references.) It is equally well established that given both a lighted and a darkened area many organisms select one or the other (Fraenkel and Gunn, 1940; for fish specifically see Schiche, 1921; Young, 1935; Breder and Rasquin, 1950; Collins, 1952 and Lowe, 1952). There are instances in which some particular intensity was selected in a gradient of light intensity (Weber, 1929; Henschel, 1929; Totze, 1933; Ullyott, 1936 and Herrström, 1949).

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[1] This investigation was assisted by a grant from the National Research Council of Canada.

The interrelations of the effects of light and temperature are clearly of interest. Some have already been described. Thus Herter (1923b) reported that house crickets selected a higher temperature in diffuse daylight than they did when exposed to lower levels of illumination, while red ants (Herter, 1923b, 1924) and flower beetles (Bodenheimer and Schenkin, 1928) selected a lower temperature in daylight than when less intensely illuminated. Actually there are apparently some instances in which no interrelation exists. Thus Herter (1923a, 1924) found that the temperatures selected by fireflies and field crickets, respectively, were independent of the incident light intensity.

The distribution of organisms in such a non-uniform environment as a temperature gradient is characterized not only by its mode, which is taken as the condition selected, but also by its spread. The latter indicates the sharpness or precision of the selection and has been measured by the standard deviation of the distribution. Herter (1923b, 1924) showed in this way that the precision of temperature selection by adults of *Acheta domestica* and *Lyogryllus campestris*, respectively, was greater (*i.e.,* the standard deviation was smaller) in light than in the dark. Although Bodenheimer and Schenkin (1928) do not discuss this point, the standard deviations of the distributions they observed for the flour beetle and for *Scanthius aegyptius* were not the same in darkness and in light. In these cases temperature selection appeared more precise in the dark than in the light.

The effect of the ambient light intensity on temperature selection by fish does not appear to have been examined, although the reverse experiment, the effect of different constant temperatures on light selection has been reported (*cf.* Andrews, 1946 and the preliminary work by Collins, 1952). Moreover, although the behavior of fish in response to simultaneously operating factors has been examined in certain connections, the distribution of fish in an environment in which there were non-uniformities of light as well as of temperature does not appear to have been studied. We have therefore determined the selected temperature of trout and the precision of selection at two light intensities and, because it was a necessary prerequisite, the precision of selection in successive experiments with the same fish; data were also obtained which establish the nature of the distribution to be expected in an environment in which non-uniformities of light and temperature are superimposed.

Materials and Methods

The experimental animals were young speckled trout, *Salvelinus fontinalis* Mitchill, two to three inches long. They were obtained from a private hatchery[2] and held in running Toronto tap water.

For all the experiments to be discussed, a five-foot long copper-bottomed trough with glass sides was used. This apparatus, the method of establishing and maintaining a temperature gradient in it and the procedure followed to determine the temperature selected by the experimental organisms have been described previously (Sullivan and Fisher, 1953).

For the experiments to be reported here the trough was provided with an arrangement of lights and opaque light shields ("covers") by means of which the desired lighting conditions were set up. A diagram showing the trough and the light shields in cross section will be found in Figure 1. The trough in which the fish were placed is designated as (a). Shield (b) was closely applied to the back of the trough (a), and effectively prevented any entrance of light from that direction. Shield (c) similarly covered the front of the trough except for a longitudinal slit along the bottom which permitted observation of the fish in the trough. Shield (d) was made of several overlapping sections so that by removing appropriate sections and adjusting the remaining ones any desired region of the trough could be illuminated. To make the boundary between the lighted regions and the dark regions as sharp as possible, vertical shields were hung from the edges of cover (d) to within ½ inch of the surface of the water in the trough.

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[2] It is a pleasure to acknowledge the cooperation of Mr. Herbert Pearen of Hornings Mills, Ontario, in this connection.

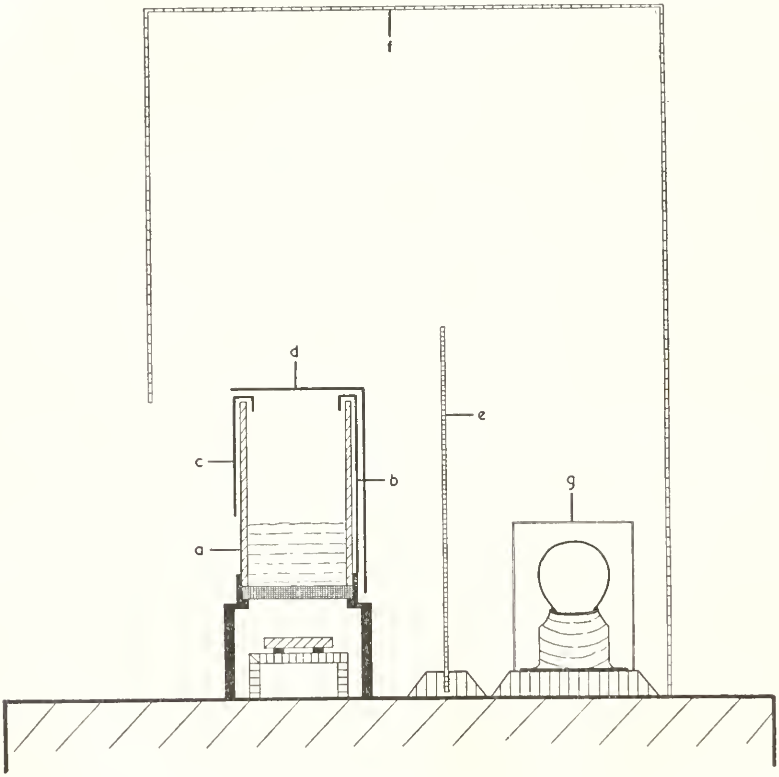

FIGURE 1. Diagram of trough and light shields; cross-section.

Light was provided by a row of six electric bulbs evenly spaced along the back of the apparatus. The illuminated surfaces of shields (e) and (f) were left their natural color (light buff) so as to provide diffuse reflection of light into the uncovered regions of the trough. All other surfaces were painted flat black.

Two types of experiments were done. For the first type, in which the whole trough was evenly illuminated, all the sections of shield (d) were removed. Under these circumstances "bright" light was provided by using 25-watt bulbs in the six sockets; "dim" light was obtained by using 7½-watt bulbs and covering the whole row of bulbs with thin white cardboard ((g) in Fig. 1).

For the second type of experiment, in which only part of the trough was illuminated, the required region was uncovered by adjusting shield (d). Six 25-watt bulbs were used. The intensity of light in the uncovered region was reduced as required by placing one or more layers of white paper over the opening in shield (d).

RESULTS

The effect of the incident light intensity on the selection of temperature may be determined either by studying a given group of fish at each of the light intensities to be used, or by using several groups of fish each of which is studied at only one intensity. The first of these procedures involves the subjection of a group of fish to two or more light intensities in succession. In it there is clearly a possibility that the behavior in the second and any subsequent exposures in the gradient may be affected by the previous experience in the gradient. A serial effect of this kind is of interest, of course, not only because it might affect the interpretation of the data obtained by procedure one, but also for its own sake, since the existence of any such serial effect would be indicative of a kind of learning by the organisms. The first experiments to be discussed here provide data about the serial effect in gradient experiments.

Effect of successive exposures to the temperature gradient

Each of nine groups of fish was studied upon three or more occasions, these being separated by one week or more. The light intensity used ("bright," see Methods) was constant along the gradient and was the same throughout all of these experiments. Typically the histogram which described the distribution of the fish in each experiment was definitely peaked. There was a considerable variation in the number of observations in the modal group from day to day and from group to group of experimental animals. It was not possible, however, to detect any consistent change in the value of the temperature selected from trial to trial other than those that normally occur with changing seasons (Sullivan and Fisher, 1953).

It seems then that as far as the actual temperature selected was concerned there was no serial effect. The sharpness of the selection on the other hand, as indicated by the standard deviations of the various distributions, did appear to change with successive determinations. These standard deviations are listed in Table I and it is evident from the mean values that the distributions tended to be spread out more on the temperature axis in the initial trial than in subsequent ones. Moreover the tendency for the standard deviation to decrease in successive trials may be seen in each of the individual groups of fish with the exception of groups 4 and 6—and it is particularly apparent in the cases of groups 1, 2 and 3, which were each observed in four successive experiments. The mean value for the nine groups in trial 1 is significantly different, in the statistical sense, from the mean value in trial 2 ($t = 2.5$, $n = 8$, probability 4%). The mean value for the groups in trial 1 is likewise different from that in trial 3 ($t = 2.6$, $n = 8$, probability 4%).

In these experiments, then, the standard deviation of the distribution of a group of fish in the gradient decreased somewhat with successive tests up to the third or fourth such tests. It is not at all apparent how this could have been an artifact resulting from the experimental conditions and so it must be concluded that it was a property of the organisms themselves. Experience in the gradient *per se* or be-

TABLE I

Standard deviations of selection histograms obtained in successive trials of nine groups of fish in temperature gradients

Group	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
1	4.38	3.48	3.53	3.02	3.02
2	4.95	5.07	4.71	4.32	
3	4.56	4.08	3.96	3.60	
4	2.94	2.91	3.09		
5	3.44	3.36	2.84		
6	4.23	4.23	4.17		
7	4.62	3.84	4.38		
8	4.62	3.99	3.75		
9	4.68	3.24	3.02		
Mean	4.27	3.80	3.72	3.68	

cause of the development in the fish of a familiarity with the apparatus led to a sharper, *i.e.*, more precise, selection of temperature.

Effect of incident light intensity on temperature selection

The experiments made to determine the precision of temperature selection in bright and dim light involved four different groups of fish, each consisting of ten individuals. Each group was divided into two lots, designated (a) and (b), respectively. Lot (a) of each group was examined first in dim light and then, in a second experiment, in bright light, while lot (b) was examined first in bright

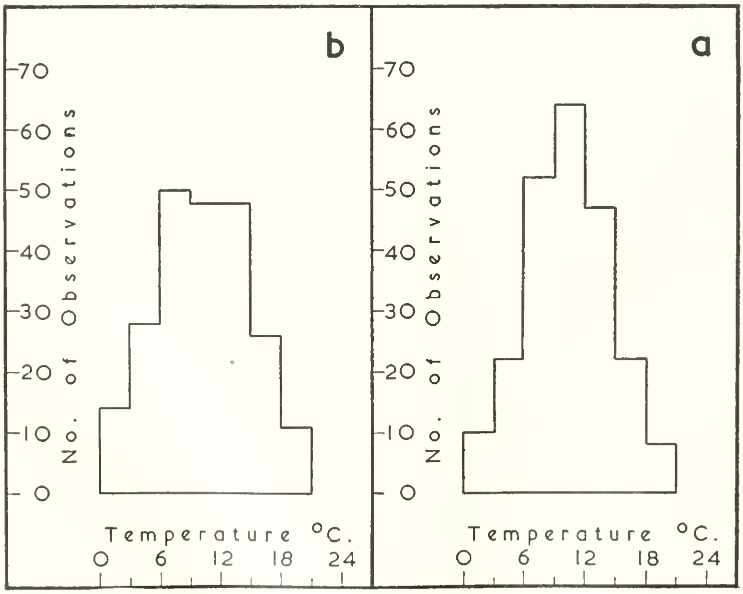

FIGURE 2. Distribution of trout in the temperature gradient. a) gradient brightly lighted; b) gradient dimly lighted.

light and then, in a second experiment, in dim light. These two determinations of the selected temperature were the only ones made on each of the two lots of groups one, two and three. A third determination was made, however, in the case of the two lots of group four, and for this determination each lot was exposed again to the initial illumination condition which it had experienced.

Histograms showing the average distribution of the organisms in dim and bright light respectively are shown in Figure 2a and b. It may be seen that a more peaked distribution occurred in the dim light (Fig. 2a). The standard deviation of the distribution in Figure 2a is 4.08 as compared with 4.62 in Figure 2b. Apparently the precision of selection was greater in the dim than in bright light. Such experiments as these which have just been discussed involved subjection to two different light intensities successively, and, as was noted above, repetition of temperature selection experiments tends to elicit sharper selection. As a consequence it could be expected that the apparent difference between the sharpness of selection in dim and bright light would be greater when the first determination was made in bright light than when it was made in dim light. In the one case, the effect of repetition would reinforce the change due to the difference in light intensity, while in the other, it would tend to cancel it. It is of interest, therefore, to examine the standard deviations of the individual distributions which were combined to produce Figure 2. They are given in Table II. The values joined by arrows were determined on the same lot of fish, the sequence of the experiments in light and dark being indicated by the direction of the arrows.

TABLE II

Group	Dark	Light	Time in gradient
1	(a) 4.11	(b) 5.46	In this series Ia and Ib were tested one day and five days later Ia was tested twice, first in light, then in dark
	3.87 ←	→ 4.98	
2	(a) 3.46	(b) 4.86	First
	3.81 ←	→ 4.23	Second
3	(a) 4.71	(b) 4.83	First
	4.02 ←	→ 4.20	Second
4	(a) 4.35	(b) 4.59	First
	3.96 ←	→ 4.56	Second
	3.72 ←	→ 3.96	Third

If one examines the instances in which the determination was made first in bright and then in the dim illumination (one instance for each of groups 2 and 3 and two instances for group 4) it may be seen that without exception the standard deviations are considerably greater in bright than in dim light. When, however, the instances are taken in which a given group of fish was observed first in dim and then in bright light, the standard deviation in the second experiment is greater than that in the first in only one instance (Group 3a). In one case (4a, second and third trials) the standard deviations are the same while in the remaining three cases the standard deviation of the first determination in dim light is actually considerably less than that of the second experiment in bright light.

As was to be expected then, the serial effect enhanced, if anything, the change in standard deviation seen when the organisms were examined first in bright light but it reduced or reversed the change due to lighting conditions when the experiments in dim light were done first.

There is then no question that a given lot of fish select more sharply in dim light than in bright light and it is evident that the serial effect operates even though the additional factor of light intensity is also involved.

These experiments in which the standard deviation of the temperature selection distribution were determined at the different light intensities also provide information about the temperature selected at the two light intensities. Nine comparisons of the temperature selected by the (a) lots at one light intensity with the temperature selected by the (b) lots at the other intensity may be made.

The pairs of values, in ° C., were as follows, the top member of each pair being the observations in bright light in each case:

13.2	13.2	11.5	8.1	12.2	10.3	10.5	7.5	7.8
13.4	13.4	10.9	8.1	10.5	10.6	10.5	7.9	9.0

The mean value at both intensities was 10.5° C. It may be concluded that the temperature selected by brook trout is not dependent on the ambient light intensity within the limits of intensity used here. The selected temperatures listed for both bright and dim light do show a trend toward lower values, because the determinations were made in the autumn when a seasonal shift in the temperature selected by trout regularly occurs (Sullivan and Fisher, 1953).

Interference between selection of light and temperature

For this part of the study two experiments, *i.e.*, determinations of the distribution of five organisms in the experimental temperature gradient, were done each day. In the first or control experiment a group of five fish was observed for two hours (five fish, each located forty-eight times, making a total of two hundred and forty observations) in the temperature gradient with the gradient trough completely covered. In this experiment the selected region was established. In the second experiment a comparable group of organisms from the same laboratory stock was used, and the region selected in the control experiment was exposed to the desired illumination. The highest intensity employed will be arbitrarily designated 100% (this corresponds to an incident illumination density of approximately 15 foot candles); other intensities will be specified relative to this. It may be recorded here that as a consequence of the particular light arrangements used in these experiments (described under Methods) the intensity of light in the darkened regions of the trough was found to be practically independent of the intensity in the lighted region. The actual intensity of the darkened parts, as well as the intensity of the whole trough when it was uniformly lighted with "dim" light was approximately 1% on the arbitrary scale.

Typical examples of the distributions found with the selected region (always taken to be one third of the length of the gradient trough) illuminated at the

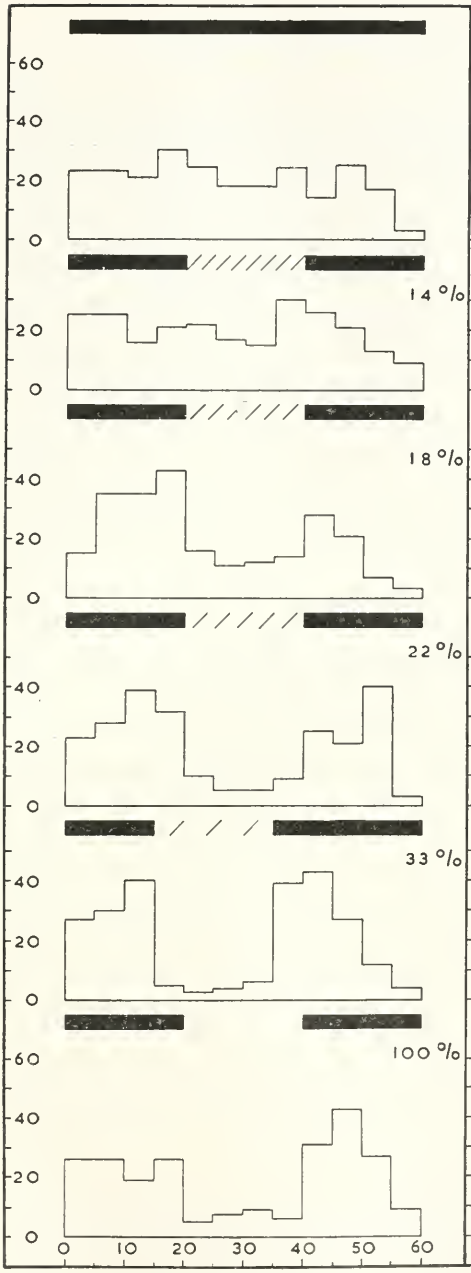

FIGURE 4.

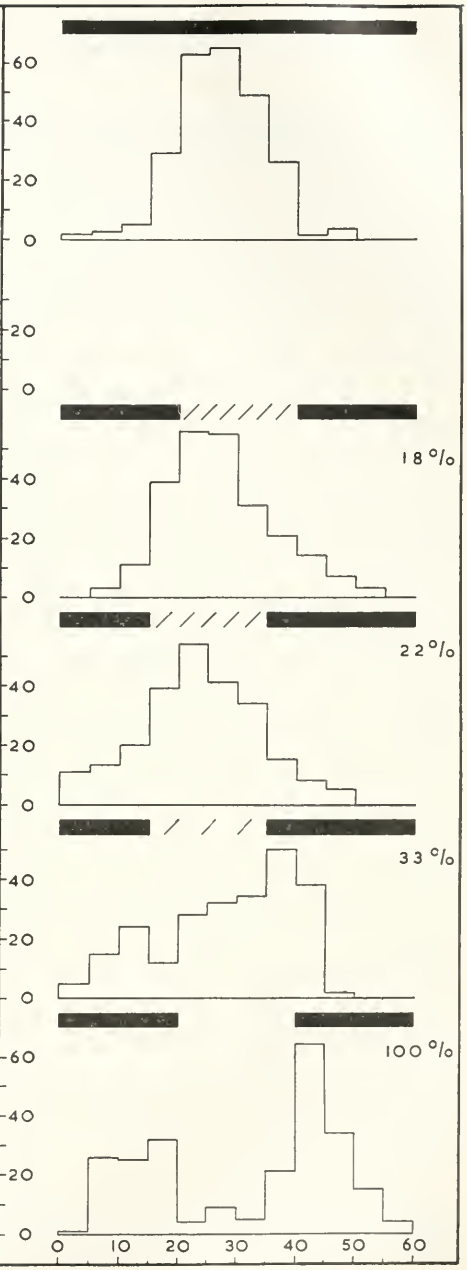

FIGURE 3.

FIGURE 3. Distribution of trout in the temperature gradient. The horizontal axis represents inches along the trough and the vertical axis represents frequency of observations of fish. Light conditions in each experiment are indicated by the black bars. Illumination of a particular region of the trough is indicated by interruption of the black bar over that region. The relative intensity of illumination of the exposed region in each experiment is given by the figure at the upper right of each distribution.

FIGURE 4. Distribution of trout in the trough at constant temperature. For further explanation see the legend of Figure 3.

intensities indicated at the right of each histogram, respectively, are given in Figure 3. The distribution at the top of the figure was found when the trough was uniformly illuminated. The black bar indicates that the light was dim, *i.e.,* the whole trough was covered. Normal temperature selection took place. The distribution shown at the bottom of the figure resulted when the selected region was illuminated with the highest intensity. With this condition, as noted above, the density of illumination in the lighted region was approximately 100 × that of the darkened region. It is apparent that this difference in intensity along the trough produced a marked effect. The region now illuminated rarely contained organisms although the latter were observed in it more frequently than elsewhere when the only non-uniformity was temperature. Those animals which did occasionally move into the lighted region characteristically swam rapidly through it to the shade on the other side. Most of the organisms were observed to stay just under the edges of the covers on either side of the lighted area, moving about quietly and thus giving rise to the distribution shown in the figure. It is evident that the reaction of the organisms to the non-uniformity of the illumination conditions was stronger than was the reaction to the non-uniformity of temperature.

With the selected region illuminated at an intensity of 33%, normal temperature selection did not occur although Figure 3 shows that the effect of the light was not as great as at an intensity of 100%. At intensities of 22% and 18%, it appeared that the light was not effective in keeping the organisms out of the selected region. Essentially normal temperature selection was found at these and lower levels of illumination.

These experiments did not show whether the fish were simply incapable of perceiving light at the lower intensities, or whether the response to light at these intensities was actually "overruled," as it were, in the central nervous system of the organisms by the reaction to temperature. To obtain information on this point some experiments were made with the trough at a uniform temperature of 11° C., the middle third of the trough being illuminated at different intensities in exactly the same way as was done when a temperature gradient was used. Data typical of those obtained in such experiments are given in Figure 4. In each case the light intensity was the same as that used in the gradient experiment which appears beside it in Figure 3. It may be seen in Figure 4 that all intensities down to and including 18% of the maximum, effectively kept the trout out of the illuminated area. At an intensity of 14% they were relatively indifferent to the light and the distribution at this intensity was not significantly different from that found with the trough uniformly darkened and at a constant temperature.

It will be noted that although the organisms stayed out of intensities 18% and 22% when the trough was at a uniform temperature, they occupied areas illuminated at these intensities in the temperature gradient when the selected temperature was arranged to occur in them. It is thus evident that the failure of light at the 18 and 22% levels to modify the distribution in the temperature gradient was not the consequence of an inability to perceive or appreciate such intensities. Instead it must be concluded that there was competition between the responses to light and to temperature in the gradient. The intensity of light which the fish would enter in the presence of a temperature gradient was considerably greater than the intensity which they would enter if no gradient were present. At high light intensities the negative response to light predominated and the trout were kept out of the selected region when it was lighted. As the intensity of light was reduced the relative effectiveness of the light and temperature stimuli on behavior changed, and at an illumination of approximately 20%, the response to temperature had come to predominate so that the animals selected temperature as though no non-uniformity of illumination existed.

The reactions to non-uniformities of light described above were interpreted as "selections" of dim light in preference to bright light. Theoretically, at least, this could have been incorrect. Instead, it might have been that in their movements the organisms tended to preserve the existing condition, dim or bright, rather than move across a demarcation line, from dim to bright, or vice versa. In other words, the fish may not actually have selected a dim light rather than a bright one. They may simply have "hesitated," as Breder (1951–52) expressed it, to move from the one condition, dim, to the other, bright. Should this be the case then it would follow that the selection of a particular region in a temperature gradient would be *less* sharp with the region in question shaded than it would be with the gradient uniformly illuminated. This, however, was not the case. When the region selected in the temperature gradient was shaded with the remainder of the trough highly illuminated, the fish selected very sharply—all remained under the cover. It must be concluded that these reactions to light are correctly interpreted as active selection of some intensities in preference to others; in general, selection of dim light in preference to bright light.

The fish used throughout the investigation reported above were exposed to the normal daily fluctuations of light intensity. The effect of eliminating these fluctuations for a period prior to such experiments as are described here was examined in two instances. For one of these a group of fish was placed in a white tank and exposed continuously for three weeks to the light of a 60-watt lamp placed about two feet above the water. For the second a comparable group of fish was kept in continuous darkness for three weeks. Each group was then tested in a gradient with the selected region illuminated and the rest of the trough darkened as usual. Both the light and the dark adapted fish were kept out of the selected region by 100% light and there was no detectable difference in the behavior or distribution of the two groups.

It would be expected that the effect of light in these experiments was mediated by the eyes. That this is in fact the case was demonstrated by experiments with blinded fish. These were found to select temperature perfectly normally even with the selected region exposed to the maximum intensity, the rest of the trough being in "darkness."

SUMMARY

1. The distribution of groups of five speckled trout in a longitudinal gradient of temperature was studied under a variety of conditions of illumination.

2. The modes of the distributions, *i.e.,* the selected temperatures, did not change with successive determinations separated by a week or more. However the selection of temperature became more precise as the fish acquired experience in the apparatus.

3. Selection of temperature was apparently more precise at low light intensities than at high intensities although the actual temperature selected did not vary with the light intensity.

4. Some experiments were done in which only part of the experimental environment was illuminated. When temperature was constant trout would not remain in the lighted part unless the intensity of illumination was very low. When, in a gradient of temperature, the illuminated part was made to coincide with the region normally selected by virtue of the temperature, it was found that at high light intensities the organisms did not appear in the illuminated region. Clearly a response to light prevailed. At intermediate and low light intensities, on the other hand, it was the response to temperature which prevailed and in spite of the illumination the organisms selected temperature in a normal way.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY
1954

ABSTRACTS OF SEMINAR PAPERS

July 6, 1954

An action spectrum analysis of the early development of Drosophila. Allen S. Goldman.

An action spectrum analysis was made in order to determine the identity, location and role of photo-inactivated compounds in the early development of Drosophila. The superficial cleavage of this egg presents two early stages in which radiation damage to the nucleus can be differentiated from radiation damage to the cytoplasm. Eggs radiated with small doses of ultraviolet when the nuclei are exposed to U.V. radiation either develop normally or show an abnormal retraction of their protoplasm resulting in complete early inhibition of their development.

Eggs with cytoplasm exposed and nuclei protected within the interior of the egg were radiated with larger doses of several monochromatic wave-lengths of the U.V. spectrum. These embryos either developed normally or showed evisceration of their yolk or herniation of their intestines. Differentiation of the rest of the embryos is apparently complete. Eggs radiated with U.V. wave-lengths from 2700–3131 Å show some nuclear damage because of the deeper penetration of the longer wave-lengths through the transparent cytoplasm. A mathematical confirmation provides evidence that the radiation damage to the cytoplasm can be differentiated from radiation damage to the nuclei.

By means of an action spectrum analysis, a quantitative assay of the spectral efficiencies in causing the abnormalities characteristic of eggs radiated with cytoplasm exposed indicates that the absorbing compounds affected are purine- and pyrimidine-containing compounds. Furthermore, since the affected purine- and pyrimidine-containing compounds must be located in the cytoplasm, a working hypothesis is adopted that cytoplasmic nucleic acid, RNA, is the affected compound and that damage to this compound leads to the observed abnormal tissue movements and presumably to abnormal tissue interactions.

Radiosensitivity with respect to the estrus cycle in the mouse. R. Rugh and H. Clugston.

Radiobiological studies with the mouse indicate that reproducible results can be achieved better by using males rather than females. This fact led to a study of survival of female CF_1 mice receiving male LD 50/30-day x-ray exposures but at different phases of the estrus cycle.

Under identical geometrical conditions of x-irradiation, litter-mate male mice exhibited a lower survival value than any females, no matter in what stage of estrus they were x-irradiated. Thus, the male CF_1 mouse is considered to be more radiosensitive than the female.

The females x-irradiated during estrus had the best survival value (66% in one experiment) as contrasted with those x-irradiated during diestrus (36% survival at 625 r). The intermediate stages of pre- and post-estrus are of short duration (6 hours) and are not easily distinguished by the vaginal smear method. These stages are critically defined as having approximately equal numbers of cornified epithelial cells and leucocytes. They were therefore considered together. In the three sets of data, involving 500 mice, the survival of these two combined stages was intermediate between the estrus and diestrus in one experiment and tended to be comparable to estrus survival in the others. This is explained on the probability that during pre-estrus there is a greater secretion of estrogenic hormones, and the proportion of pre- and post-estrus mice in the three experiments would vary.

While there is a correlation in total body weight of the female mouse with the phases of estrus, suggesting hydration changes with maxima during diestrus, this is not considered as the primary factor attributing to the female cyclic changes in radiosensitivity. Rather, periods of greatest radiosensitivity are correlated with estrus, and it is believed that the elaboration of estrogens provide a chemical source of protection. This concept is supported by experimental data in protection studies.

The relationship between the surface structure and the electrophoretic mobility of E. coli bacteria. Charles C. Brinton, Jr., Anne Buzzell and Max A. Lauffer.

Microscope electrophoresis studies revealed that a variant of *E. coli* B bacteria showed either organisms with a mobility of approximately 2 microns/sec./volt/cm., or organisms with a mobility of approximately 3.6 microns/sec./volt/cm. in a medium with ionic strength .018 buffered to pH 7.0 with phosphate salts. Intermediate values of mobility were not observed, but the relative numbers of fast and slow organisms varied from culture to culture. Bacteria selected for resistance to T_1 bacteriophage were found to be uniformly fast.

In seeking an explanation for the difference between fast and slow organisms, it occurred to us that a difference in the friction coefficient of the particle might be responsible. The intrinsic viscosities of a concentrated suspension of fast organisms and of a concentrated suspension of slow organisms were measured. The value for the slow organisms was found to be 3.3 times as great as that of the fast. It was reasoned that such a high intrinsic viscosity could be brought about by surface irregularities. Electron microscopy confirmed this by revealing on the slow organisms filaments about 150 Å in diameter and of length up to a micron or more, while the fast organisms were without such filaments. The filaments could be removed by agitating with a Waring Blendor; previously slow organisms became electrophoretically fast. De-filamented organisms were viable, and, upon incubation and multiplication, filaments were produced and the organisms again became slow.

The difference between filamentation and non-filamentation may be related to the difference between rough and smooth colony form. Bacteriophage susceptibility and resistance is associated with the presence or absence of filaments.

July 13, 1954

The dry matter content of starfish oocyte nucleoli. W. S. Vincent and A. H. Huxley.

The dry matter content of starfish oocyte nucleoli isolated in distilled water was determined by microinterferometry. The dry matter of nucleoli 4 μ in diameter averaged 85% (calculated as protein). With growth of the nucleolus the dry matter content decreased linearly with increasing nucleolar diameter, attaining a value of 40% in mature nucleoli 20 μ in diameter. Vacuoles in the nucleoli, occupying up to 50% of the total volume of the mature structure, contained less than 5% dry matter. Fragments of the same ovary from which the nucleoli were isolated were fixed and sectioned, and the concentration of protein in the intervacuolar areas of the nucleoli determined by microphotometry of the bromphenol blue and Millon stains. No change in concentration of protein in the intervacuolar regions of nucleoli 4 μ to 20 μ in diameter could be detected.

From these data we conclude that the starfish oocyte nucleolus is composed of highly concentrated, predominantly protein, materials in which are interspersed vacuoles containing less than 5% dry matter. Growth of the nucleolus appears to result primarily from accumulation of protein with a smaller contribution from the increase of vacuole volume.

Isolation of nuclei from Asterias eggs in the germinal vesicle stage and their DNA content. CELIA MARSHAK. No abstract submitted.

A theory of the biological role of DNA. ALFRED MARSHAK. No abstract submitted.

JULY 20, 1954

Influence of pH on the rate of respiration in Chlorella. E. P. NIELSEN. No abstract submitted.

"Univalent" antibodies. ALBERT TYLER.

Earlier experiments (1941 et seq.) showed that the fertilizin of sea urchin eggs could be converted by various methods into a non-agglutinating form termed "univalent" that retained its specific reactivity with sperm. Extension of the investigations (1945) to immune sera (rabbit anti-pneumococcus, anti-sheep cell, horse anti-diphtherial toxin, etc.) showed that photo-oxidation was an effective method of rendering the antibodies of these sera non-agglutinating or non-precipitating. In these experiments the ability of the treated antibodies to combine with antigen was tested primarily by the inhibition of a subsequent visible reaction to the addition of untreated antibodies. Another method of detecting non-agglutinating antibodies was later described by Coombs for the Rh system. This consists in adding an anti-human globulin serum to Rh-positive cells which have reacted with non-agglutinating human anti-Rh antibodies. Since the antibodies are globulins, then, having combined with the cells, the latter will be agglutinated by the anti-globulin serum.

If the antibodies that have been made univalent by photo-oxidation still retain their globulin specificity, the Coombs' test should be expected to be applicable to these, too. Experiments in collaboration with Dr. R. R. A. Coombs and M. Lorraine Fiset of Cambridge, England showed this to be the case. Rabbit anti-sheep cell sera were exposed to visible light in the presence of 0.2% eosin Y (water-soluble) until agglutinating action had been destroyed, the treatment being controlled also by measurement of O_2 uptake. Sheep cells upon treatment with such sera can be agglutinated by a goat anti-rabbit globulin serum. The univalent antibodies can also be conjugated with other substances, such as ovalbumin, and when now combined with the sheep cells, these can be agglutinated by an anti-ovalbumin serum. In addition, univalent antibodies against ovalbumin could be detected by the use of an antiglobulin serum. This procedure thus provides further opportunity for exploring the presence and role of univalent antibodies in allergy and pathogenic disease, and of investigating many other features of antigen-antibody reactions.

The activation of human plasminogen by streptokinase. WALTER TROLL AND SOL SHERRY.

Human plasma contains a proteolytic enzyme precursor termed plasminogen or profibrinolysin. The activation of this precursor to plasmin or fibrinolysin can be carried out by physical agents or specific bacterial kinases, *e.g.*, streptokinase. Two views have been proposed for the mechanism of the activation of plasminogen by SK. According to one view plasminogen is converted to a new substance, plasmin, by enzymatic activation. According to the other view the conversion of plasminogen to plasmin results from a stoichiometric interaction of SK with plasminogen. Our data reconcile the observations of these investigators. With the aid of synthetic substrates we have obtained evidence that plasminogen preparations contain two factors. One of these is activated by SK in a stoichiometric fashion to yield a plasminogen activator which enzymatically converts the second factor, plasminogen, to plasmin. The plasminogen activator has the properties of an enzyme splitting lysine esters, but is without action on arginine esters or casein. Plasmin, however, splits arginine and lysine esters and is proteolytic on casein.

JULY 27, 1954

Sympathectomy in frogs. JOSEPH PICK. No abstract submitted.

Sodium and the release of acetylcholine from sympathetic ganglia. O. F. HUTTER AND KRISTA KOSTIAL.

In suggesting the hypothesis that acetylcholine may be released from the nerve endings in exchange for sodium, Fatt and Katz called for verification by measurement of acetylcholine output. We have made an attempt in this direction by assaying the quantities of acetylcholine released from sympathetic ganglia perfused at various sodium concentrations.

Superior cervical ganglia of cats were perfused with eserinized Locke's solution, sodium being replaced by sucrose. So long as the perfusion fluid contained enough sodium for nerve impulse conduction no change in the output of acetylcholine could be detected. For example, stimulation of the preganglionic nerve for 5 minutes at 2 per second released as much acetylcholine after perfusion for ½ hour with a solution containing 30% of the normal sodium concentration, as when perfusing with Locke's solution. When the concentration of sodium was reduced to levels at which nerve conduction fails the output of acetylcholine decreased suddenly.

In some experiments ganglia were perfused with solution in which all sodium was replaced by sucrose, and the effect of depolarizing the nerve endings by increasing the potassium concentration was tested. The amounts of acetylcholine released by potassium under such conditions were comparable with the amount released by potassium in the presence of sodium.

These results indicate that in sympathetic ganglia sodium plays no part in the release of acetylcholine. There are, however, important differences between the anatomy of the nerve endings in ganglia and in muscle, and it is not impossible that our results are due to the presence of a diffusion barrier around the preganglionic nerve terminals which prevents the exchange of sodium for sucrose.

These experiments were made at University College London and at the Institute za Medicinska istrazivanja, Zagreb.

Electron microscopy of the sarcosomes in the heart. BRUNO KISCH.

The author emphasized first in 1951 the vital importance of the sarcosomes in the heart, being bearers of metabolic enzymes and responsible for the ability of the heart to work during a lifetime without longer rest. Biochemists all over the world have since proven the presence of these enzymes in the sarcosomes of the heart and the skeletal muscle.

Pictures of the inner structure prove a lamelliform arrangement of material. Sarcosomes of the mitochondrial type seem to prevail in the frog heart but not in the heart of mammals. The lamels are either crossing the sarcosomes in parallel layers or are arranged in concentric shell around one or different centers, or they are folded like a curtain. In some sarcosomes the content in cross-section looks like rings or loops or doughnuts.

A change of the appearance of the inner structure in connection with the functions of the sarcosomes is supposed, and attention was called to the startling similarity of the lamelliform contents of the sarcosomes and the arrangement of the myofilament in the muscle fibrils to Otto Lehmann's liquid crystals. After death the content of the sarcosomes disintegrates and more or less empty bags, the "ghost of sarcosomes," or their shadows, similar to the ghosts of red blood cells, remain, showing ridges or lamels inside a membrane not regularly arranged.

A not yet known new structure in the muscle fiber of the heart and the skeletal muscle was shown: a tube or fiber of ca. 300–500 Å width, straight or branching out between the myofibrils.

AUGUST 3, 1954

Identifying gene elements in Mormoniella. P. W. WHITING.

Of the many recessive eye-color mutant types found in this wasp, the majority occur at a single locus, R, consisting of two mutable elements, O and S. If either simple dominance or blending occurs in the females compound for two mutant colors, the two mutations have affected the same element, either O or S. Codominance, complementarity, indicates that the two mutations have affected different elements, O and S, each change being recessive to its wild-type alternative in the other allele. A mutant male to be tested is crossed to a wild-phenotype female, compound for recessives in different elements, O.s/o.S. From the two different types of unfertilized eggs, O.s and o.S, laid by such a tester female there develop the two expected types of males. From the fertilized eggs there are produced females of two genotypes, both of which bear the new mutant paternal gene, m. If both female genotypes are wild phenotypes, O.s/O.S +/m and o.S/O.S +/m, the new mutation is not in R. If half the females are wild-type, the new mutation is in R, either in O, the females being O.s/m.S (wild phenotype) and o.S/m.S (recessive phenotype) or in S, the females being O.s/O.m (recessive phenotype) and o.S/O.m (wild phenotype). If none of the females are wild-type, the new mutation involves both elements, the females being O.s/m.m and o.S/m.m (both recessive phenotype). The fact that no R-locus mutants have been found siring only wild-type daughters suggests that there are not more than two mutable elements at this locus.

Some aspects of sexuality and genetics in desmids. Richard C. Starr. No abstract submitted.

On inherited and adapted rutin-resistance in Chlamydomonas. Franz Moewus.

Three strains of *Chlamydomonas eugametos* have been used in this investigation: *eugametos* (f. *typica*), characterized by a highly potent sexuality; *agametos*, characterized by its complete sterility; mutant *reru*, derived from *typica*. It could be shown that the sterility in *agametos* is due to a false biosynthesis of the flavonoid hormones, leading to the final product rutin instead of isorhamnetin or peonin. Rutin could be isolated from *agametos* cells. If rutin is offered to *eugametos* (10^{-10} g./ml.), it sterilizes completely this highly sexual strain. However, it has no sterilizing effect on the *reru*-mutant. In homogenates of *reru*-cells an enzyme system could be demonstrated which converts the offered rutin to the harmless ombuoside (=7.4′-dimethylrutin). These results are only valid if the strains are grown under our usual standard methods. Their essential point is that the experimental material is obtained by spreading a suspension of *motile cells* on agar, where they undergo a log-phase of growth and develop within 2–3 weeks a monocellular film. Different results are obtained, when *eugametos* and *agametos* are grown by spreading thick layers of *non-motile cells* on agar, where they develop a slow growth. After one or more passages on agar (each time transferring the whole mass of non-motile cells onto the next plate), the physiological behaviour of *eugametos* and *agametos* is changed. They are characterized by heavy production of mucilage: they have become palmelloid. Palmelloid *agametos* shows now normal fertility, palmelloid *eugametos* is now rutin-resistant. In homogenates of both palmelloid strains the same enzyme system as in *reru*-mutant could be detected. In *agametos* the naturally produced rutin, in *eugametos* the offered rutin is converted to the harmless ombuoside. The appearance of this rutin-resistance is probably not due to spontaneous mutation. Experiments are in progress in order to explain this adapted character.

August 10, 1954

Ionic distribution and glycolytic factors in Fundulus. D. R. Shanklin.

Because of their relative abundance at Woods Hole, developing *Fundulus* embryos between Oppenheimer stages 22 and 27 were utilized in studies on cationic balance. During these stages there is a covering of ectoderm without orifices; circulation is complete and ectodermal-axial reflexes provide vital signs for biologic controls. During these stages the yolk occupies 50–60% of the total volume and is readily separable for distribution studies. Analyses indicate that magnesium is the principle intracellular ion with an inwardly directed gradient; sodium, potassium, and calcium having outwardly directed gradients. Yolk contains more free calcium than the embryo proper and less of the others; fluoride affects only the calcium content in yolk.

Bound fractions were determined. Influx of ions is linear, and at differing times for each ion, a plateau of exchangeability saturation is reached. From this plateau loss studies into sea water and osmotically balanced media lacking the ion in question were done. These losses are linear. Studies of this system with glycolytic inhibitors fluoride and iodoacetate were done, yielding changes in the slopes of uptake and loss, but not in the linearity. Since diffusion curves are hyperbolic, the linearity of these responses suggests a limiting transport system in both directions. Inhibitor studies show glycolytic tie-up with all four ion balances, the action towards sodium transport being in the direction of the gradient. A comparison of the potential maximum influx rate with the potential maximum outflux rate, measured against null gradient, in all cases yields a ratio statistically equal to the gradient, which would be expected if the cell were the cause of the gradient.

Some properties of mature and young erythrocytes. DAVID CHALFIN.

A comparison of red blood cell changes *in vitro* and red cell aging *in vivo* was undertaken. Erythrocytes from normal rabbits were incubated under bacteriological sterile conditions at 37° C. and followed for 30–40 hours for the *in vitro* studies. For the *in vivo* measurements, erythrocytes taken from a rabbit which had been rendered anemic by repeated hemorrhage were fractionated. The anemic blood was centrifuged and most of the reticulocytes were found to be concentrated at the top (centripetal portion) of the packed column of cells whereas the older cells were found at the bottom (centrifugal end) of the tube. Measurements were made on cation content, osmotic resistance, cell volume, hemoglobin content, density, dry weight and per cent water per cell for young and older red cells.

The younger red blood cells of rabbit are about one and half times larger, have a greater osmotic resistance, a lower density, a greater dry weight, and a higher K content than do older erythrocytes. The Na content remains relatively unchanged. The hemoglobin content remains constant as the cell matures but the cell volume decreases. The cation changes observed accompanying *in vitro* cell destruction: loss of K, gain of Na, loss of cation gradients, and increase in cell volume, are not the same as *in vivo* aging of the cell. Aging cells show a decrease in dry weight, a decrease in cation content as well as a decrease in cell volume, but a maintenance of the cation concentration and, most important, a maintenance of the cation gradients across the cell membrane.

Some new permeability constants for erythrocytes and their possible significance. M. H. JACOBS.

Under conditions and by procedures discussed in detail elsewhere it is possible to obtain for erythrocytes permeability constants (P) which measure the amounts of dissolved nonelectrolytes that cross unit area of the cell surface in unit time with unit difference of concentration between the inside and the outside of the cell. Approximately 100 such constants have been determined involving 16 species of mammals and 8 solutes. Erythrocytes of the groundhog and the muskrat are sharply separated from those of the other species and from most other known cells by P values, near 20° C., in cm./hour $\times 10^4$, of the order of several hundred, for all the solutes studied. Erythrocytes of the goat, sheep, beef, pig, horse, cat, dog, macaque, and chimpanzee showed values ranging from 0.4 to 3.9 for glycerol, 22 to 176 for ethylene glycol, and 6 to 51 for thiourea. These values are very similar to those reported by others for cells of about a dozen species of higher plants. Erythrocytes of man and the rat, rabbit, guinea pig and mouse showed values for glycerol from 12 to 120 for glycerol and 215 to 680 for ethylene glycol, but were very similar to those of the preceding group with respect to thiourea. The values for urea in the case of all species ranged from about 5,000 to 10,000, which may be compared with the average value of 3.7 for cells of 12 higher plants. For all the erythrocytes except those of the groundhog and muskrat the diethylene glycol and triethylene glycol values were fairly close to the averages for the species in question of 27 and 55, respectively. P values for diacetin were all of the order of several hundred and those for monoacetin, with a few exceptions, lay between those for diacetin and glycerol.

August 17, 1954

Activation of stretch receptors in Crustacea. Carlos Eyzaguirre. No abstract submitted.

Effect of sympathetic stimulation on the slow skeletal muscle system of the frog. O. F. Hutter and W. R. Loewenstein.

In most skeletal twitch muscles of the frog, neuromuscular transmission proceeds, as a rule, with a considerable margin of safety: so long as the end-plate potential is large enough to set up a propagated action potential the muscle fibre gives a twitch. In such muscles sympathetic nerve activity can play a role only if a fringe of subliminally excited muscle fibres exists. The contractile mechanism of the multiply innervated slow skeletal muscle fibres of the frog is, however, activated in a different manner. Propagated action potentials are absent; instead there appears to be a close relation between the state of depolarization of the membrane and the contraction of the fibre. Since many similarities exist between the twitch fibre and the slow fibre system of the frog, it seemed likely that an agent facilitating neuromuscular transmission in twitch fibres will increase the tension response of unfatigued, untreated slow fibres. We have found that sympathetic stimulation increases the tension produced by the slow skeletal muscle fibres in iliofibularis on excitation of the small motor nerve fibres in the ventral roots.

Nature of neuromuscular facilitation by sympathetic stimulation in the frog. O. F. Hutter and W. R. Loewenstein.

It has long been known that in fatigued frog muscle stimulation of the sympathetic fibres causes a partial recovery of the twitch tension. This phenomenon has been ascribed to the diffusion of an adrenaline-like substance from the nerve endings of the vascular sympathetics to the muscle fibres. Since the twitch tension does not increase if the muscle is stimulated directly, it has been suggested that sympathetic stimulation causes a partial relief of neuromuscular block. Recording of muscle action potentials confirms this conclusion. In preparations partly blocked by fatigue or by too little initial stretch or by tubocurarine, sympathetic stimulation causes an increase in the voltage of the compound muscle action potentials, indicating the recruitment of fibres that have previously failed to respond.

In fully curarized preparations sympathetic stimulation, adrenaline and nor-adrenaline cause an increase in the amplitude of the end-plate potential. The increase rarely exceeds 10% but it can account for the observed restoration of transmission. The time course of the end-plate potential and the membrane potential of the muscle fibre are not detectably altered.

To localize the mechanism responsible for the neuromuscular facilitation the effect of nor-adrenaline on the depolarization of the motor end-plates produced by applied acetylcholine was studied. In a concentration of 2 in 10^6 nor-adrenaline increases the depolarization produced by a constant dose of acetylcholine by about 20%. It may be inferred from this that the increase in the nerve elicited end-plate potential is also due to a sensitization of the motor end-plates to acetylcholine.

In vivo measurements of the squid giant axon resting and action potentials. K. S. Cole and J. W. Moore. No abstract submitted.

GENERAL SCIENTIFIC MEETINGS

August 23–26, 1954

Abstracts in this section (including those of Lalor Fellowship Reports) are *arranged alphabetically by authors* under the headings "Papers Read," "Papers Read By Title" and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index (published in the December issue).

PAPERS READ

A new fibrous muscle protein. W. R. AMBERSON, J. I. WHITE, H. B. BENSUSAN, S. HIMMELFARB AND B. BLANKENHORN.

Extracts of rabbit skeletal muscle contain, in addition to actin and myosin, a third fibrous protein, provisionally called D-protein, which unites with myosin to form a complex, D-myosin. This complex can be detected on descending limbs of electrophoretic patterns given by mixtures of D-protein and myosin. It forms an elevation between faster moving free D-protein and slower moving free myosin. D-protein is obtained from ascending limbs by electrophoretic fractionation. Its solutions exhibit high viscosity and flow birefringence.

Solutions containing both D-protein and myosin have been studied in the Spinco analytical ultracentrifuge. The complex, D-myosin, is revealed by the use of the synthetic boundary cell introduced by Pickels, Harrington and Schachman. The lower two-thirds of the sectoral cavity is filled with a solution containing 0.4% D-protein and 0.4% myosin. The cup contains 0.4% D-protein alone. At about 8,000 r.p.m. this solution transfers from cup to sector, forming a layer which fills the upper third of the cavity. The run proceeds at 59,780 r.p.m. Two boundaries are initially seen, the upper due to D-protein alone, the lower to myosin alone. The lower boundary soon divides into two elevations. Free myosin sediments more rapidly. Myosin bound to D-protein sediments more slowly. After long running the upper D-protein boundary may reach and pass the more slowly sedimenting D-myosin elevation. A zone then exists which contains D-myosin alone, in the absence of both free myosin and free D-protein.

The strength of the union between D-protein and myosin is further demonstrated when the mixture in the lower part of the sector also contains 0.2% actin. The actin unites with all free myosin, forming actomyosin, which sediments rapidly. The elevation formed by D-myosin appears as before, sedimenting slowly. Actin is not able to cause the dissociation of D-myosin. Interactions involving all three fibrous muscle proteins may occur in the contraction-relaxation cycle.

Gel-formation in Limulus serum caused by a bacterial toxin, a protective mechanism of the host. F. B. BANG.

The intravascular clotting, and lethal effect, of a bacterial infection of Limulus was reproduced by a cell-free extract of boiled bacteria of this strain. The toxin caused a reduction in the number of cells in the peripheral blood of lobsters, spider crabs and in the coelomic fluid of Arenicola. Larger amounts killed lobsters and fiddler crabs.

Serum obtained from clotted blood in Petri dishes formed a gel within ten minutes after adding dilutions of "toxin," or living bacteria from the disease strain. Phase microscopy showed the bacteria to be immobilized by dilutions up to ⅛ of the serum. The gel was stable at room temperature for more than three weeks. Gels were formed by sera from which the hemocyanin was removed by centrifugation (35,000 r.p.m. for 6½ hrs.), but were not produced by heated sera (55° C.–10 min.). No young and not all old Limuli furnished sera which formed gels, but viscometry indicated quantitative rather than qualitative differences.

The bacterial toxin is non-dialyzable, and a similar effect was produced by a Shigella toxin of vertebrate origin.

Analysis of the luminescent responses in Mnemiopsis upon stimulation. JOSEPH J. CHANG.

The luminescent cells in the eight meridional canals of the ctenophore, *Mnemiopsis leidyi*, respond only upon stimulation, either through nerves or directly. Using photomultiplier tube, amplifier, cathode-ray oscilloscope, and camera combination, the luminescent responses to various single and repeated electrical stimuli at room temperature as well as at other temperatures have been studied.

A large piece of luminous tissue responds by multiple flashes after a single stimulus when recorded locally. However, the response characteristics measured by light production from a small bit of tissue greatly resemble those of muscle twitches. A single square wave stimulus elicits a single flash response. An increase in the stimulus voltage gradually increases the flash intensity. The same is observed when the duration of the stimulus is increased. At 23° C. a single flash curve has a latent period that varies considerably, a half-rise time of 35, a full-rise time of 60, a half-decay time of 48, and a 0.9-decay time of 114 milliseconds. Raising the temperature shortens the time course of a flash and lowering the temperature has an opposite effect. The decay of the flash intensity is logarithmic and the log decay constant vs. 1/T (Arrhenius plot) gives a straight line. Responses analogous to the summation of twitches, treppe, complete and incomplete tetanus of muscle are obtained by repetitive stimuli. Fatigue caused by multiple stimulation is very rapid in this luminous system. The luminescent response will traverse the length of a meridional canal with a conduction speed which averages 14 cm. per second at 23° C. Action potentials of fast frequency appear simultaneously with the luminescent response. Potential changes without a flash response have also been recorded along the canal.

Evidence for protein turnover in Amoeba proteus. Adolph I. Cohen and Suk Ki Hong.

Amoeba proteus in log phase growth were labelled by being fed S^{35}-labelled Chilomonas paramecium. The amoebae were then fed for 48 hours with unlabelled *Tetrahymena pyriformis.* After this period, sister cell pairs were isolated and one member mounted for radioassay. The corresponding sister cell was then allowed to feed on unlabelled Tetrahymena and after 4–5 cell generations, the total population resulting was mounted as a unit. After assaying, planchettes were extracted with cold 5% trichloracetic acid for one hour, dried, and re-assayed. The results showed a significant loss of total activity in both the non-extractable and extractable fractions during growth, while the percentage of activity that was non-extractable rose. Since cell death does not occur, turnover of some non-trichloracetic acid-extractable material or secretion of some sulphur-containing compound must account for these data. The chemical nature of the lost sulphur-containing compound is, as yet, unknown. Secretion of materials by amoebae is unknown. Preliminary experiments on starving amoebae show slight loss of total activity, if any, and less incorporation into the non-extractable fraction. Preliminary studies comparing starving nucleate and enucleate amoeba halves after six days, show a loss of activity for the nucleate halves. The enucleate amoebae show little loss, if any, and both halves show no significant incorporation into non-extractable material. Comparisons are complicated by the fact that heavily feeding and starving amoebae are far less motile than moderately fed or slightly starved amoebae. Enucleate and dividing amoebae are least active of all.

The contribution of the sperm aster to the first mitotic division. Ivor Cornman.

Mazia has demonstrated that the sperm aster, like cleavage asters, can be isolated by the technique that yields free spindles. From the similarity of material in these structures it is reasonable to conclude that the substance of the sperm aster contributes to the achromatic figure of the first cleavage. Evidence from colchicine-poisoned eggs supports this. If, by early treatment of *Arbacia* eggs, the sperm aster is prevented from forming, cleavage on removal to sea water is delayed much more than if treatment is begun after the streak has formed. In the latter case the streak material remains at the center of the egg and appears to be assimilated into the mitotic spindle on removal from colchicine. In *Asterias* eggs, where a threshold dose of podophyllotoxin has suppressed the sperm aster, the achromatic substance first materializes as many scattered spots around which there is subsequent multiple cleavage. A source of spindle material may well be the germinal vesicle, or even the nucleolus. There is enough protein in the nucleolus to form the achromatic figure and the nucleolus has solubility properties similar to those of the spindle (Vincent). Thus, the nuclear or nucleolar material is released when the germinal vesicle breaks down. The maturation divisions, unaided by the sperm aster, are able to muster only small spindles. The sperm aster, radiating throughout the egg, assembles the necessary nuclear and cytoplasmic components into the streak material at the center of the egg, where it is incorporated into the mitotic figure. The prominence of mitotic asters in large eggs undergoing holoblastic cleavage may also reflect this need for a system of collecting spindle material in an amount adequate to divide the entire protoplast.

Reconstitution of the intestinal tract in adult frogs, Rana pipiens. CHAUNCEY G. GOODCHILD.[1]

Experiments in which host organs were surgically altered to provide unusual implantation sites for parasites revealed that severed intestinal tracts of frogs possess ability not only to rejoin, but also to regain functional integrity. To study nutritional plasticity of trematodes, recta of frogs were disjoined from the ileum, washed free of debris, and used as implantation sites for gorgoderine trematodes. Operative procedure consisted of making a short incision on the right flank and lifting out the ileum and rectum. The ileum was tied two mm. anterior to the ileocolic valve and transected ahead of the stricture. The rectum was returned to the coelomic cavity and the ileum stitched to the flank to form an ileostomy. Although the ileostomy could be kept open by periodic attention, spontaneous healing by skin overgrowth usually occurred. When, in the latter, functional activity was indicated by appearance of normal fecal pellets, approximately two weeks after the operation, a series of operated frogs was studied. Even though the rectum and ileum had been purposely separated during the operation, after 5–6 days the short ileorectal stump was found adherent to the side of the ileum approximately 4 mm. from the body wall. During the next 7 days a gradual perforation of the ileum occurred at this point, and with concomitant closure of the ileostomy, the gut was reconstituted. The thread which had been used to tie the ileum was recovered in the rectal lumen. In other frogs simultaneous ileostomy and colostomy were performed in the same flank incision. In these animals, functional reunion between disjoined ileum and rectum was established within 12 days by a slight sinking in the openings and closure by a drawstring-like fold of skin growing inward from the margin. Specimens have been preserved for future histological examinations.

Survival of bladder flukes in new habitats. CHAUNCEY G. GOODCHILD.[1]

Anuran bladder flukes of the genera *Gorgoderina* and *Gorgodera* will survive when transplanted into urinary bladders of anuran species not naturally infected, but they quickly succumb when homoplastically or heteroplastically placed elsewhere in anuran hosts. Survival after implantation into urinary bladders of turtles and salamanders has now also been studied. Seven female painted turtles, *Chrysemys picta*, had 22 *Gorgoderina attenuata*, and 80 *Gorgodera amplicava* implanted into the true urinary and accessory urinary bladders. Flukes were not recovered from the latter after 24 hours. In the true urinary bladder some were recovered after 10 days; these were normal in behavior and were found only near the point of emergence of the bladder stalk. In the newt, *Triturus viridescens*, an intestinal immunity prevents bladder infection from ingested metacercariae, but implanted flukes were normal after an intravesical existence of two days. Fifty specimens of *Rana pipiens* were surgically altered to provide new implantation sites for *Gorgoderina attenuata*, taken from the same species of host. The majority of operations consisted of: 1) simple ileostomies, with the disjoined rectum forming an internal blind sac, 2) simultaneous ileostomies and colostomies, where the colon had anterior and posterior openings to facilitate cleansing and implantation, and 3) simple, midventrally-opening, small intestinal caeca separated from the rest of the gut. *Gorgoderina* placed in rectal caeca and in small intestinal caeca failed to live for 24 hours, but in recta open at each end some flukes lived in artificial frog urine for four days. Although bladder flukes failed to survive indefinitely, the new technique should be extremely valuable for elucidating host-parasite relationships of organisms regularly living in these gut regions.

Ca-initiated precipitation of nucleoprotein from homogenates of sea urchin eggs.[2] PAUL R. GROSS.

The role of calcium ions as initiators of cytoplasmic sol-gel transformations has been investigated at a biochemical level in homogenates of sea urchin eggs. The Ca^{++} is added to homogenates made from eggs previously freed of their calcium. Water extracts of such homogenates show marked differences in protein and nucleic acid content dependent upon 1) presence or absence of calcium and 2) the amount of Ca added and the time of incubation after calcification. In these experiments, the fraction of the homogenate containing the large pigment granules was removed by centrifugation at 0° C. for 10 minutes at 1000 × g. Experiments were performed upon the supernatants which contain yolk as well as all of the smaller particles.

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[1] This investigation was supported by a grant from the McCandless fund of Emory University.

[2] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

The precipitation of protein is a first-order reaction. With Ca added to a final concentration of 0.010 *M*, a homogenate may lose 10% of its water-soluble protein within 15 minutes and 18% within 60 minutes. PNA, as determined by the orcinol reaction, precipitates more rapidly, reaching a level of 26% precipitated within 60 minutes. It is assumed as a working hypothesis that the precipitated material is a nucleoprotein, richer in PNA than is the homogenate as a whole. The traces of DNA present in the homogenate cannot contribute significantly to these effects.

Electrophoretic experiments indicate that the material precipitated comes mainly from the largest fraction of the water-soluble proteins, and that this material has an unusually high mobility (-13×10^{-5} at pH 7.6 and ionic strength $= 0.1$). The high density of negative charges also suggests that the precipitating material is a nucleoprotein.

Electron micrographs of whole homogenates and of the two fractions show that the aggregating particle is 400 A.U. in diameter. These particles can dissociate into spheres of high electron density 60 A.U. in diameter.

The calcium, as measured by the method of Fales (1953), is not precipitated to a measurable extent with the macromolecular materials.

Some observations on "plasma clots" and "fibrin clots." [1] PAUL R. GROSS, ALVIN M. KAYE, DELBERT E. PHILPOTT AND PETER RIESER.

In the terminology adopted by Lorand, blood clots appear in one of two forms; one, produced by the action of thrombin on fibrinogen in the absence of calcium and/or plasma factors, is urea-soluble. This is the "fibrin clot." The "plasma clot" forms when calcium and a plasma factor are present, and is urea-insoluble.

The plasma clot is generally more opaque than a fibrin clot. This fact has led us to seek differences in clot structure at the submicroscopic level.

For these experiments, undiluted citrated sheep plasma, as well as plasma diluted 1:6 with 0.9 per cent NaCl, was clotted with either thrombin (10 units/ml.) or $CaCl_2$ solution (2 mg. Ca/ml.). For the electron microscopic observations, collodion-filmed nickel grids were dipped for 15 seconds in the clotting mixture and fixed in 0.2 per cent phosphotungstic acid at different times relative to the time of clotting in the original vessel. Afterwards, the material was washed in distilled water.

On only one occasion were large fibrin fibers showing the characteristic 230 A.U. periodicity obtained. This system was one clotted with thrombin and was more dilute than the systems described above.

Ordinarily, no periodicity was shown in the clots. Both the fibrin clots and the plasma clots were tight networks of fibrils of smaller diameter than those observed in clots formed from purified fibrinogen.

The fibrin clots were usually fine networks, and showed the substrate uniformly coated with material. The plasma clots were always more strongly cross-linked, showed marked syneresis on the substrate, and had areas of nodular, highly electron-dense material which were never found in the Ca-free systems.

X-irradiated plasma (100,000 r) produced fibrin clots whose rate of dissolution in 5 *M* urea was markedly accelerated.

A further study of isometric mechanical responses of the anterior byssus retractor muscle of Mytilus edulis to electrical stimuli and mechanical stretch. WILLIAM H. JOHNSON. See abstract under "Lalor Fellowship Reports."

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[1] This investigation was supported by a research grant from the U. S. Atomic Energy Commission, administered by L. V. Heilbrunn.

The nature of the oxygenation reaction in hemocyanin. THEMIS ASKOUNIS KLOTZ AND IRVING M. KLOTZ.

The consensus (Redfield, 1933, 1950) has been that copper exists in the cuprous state in hemocyanin, both in the deoxygenated and oxygenated forms. It has been pointed out (Klotz, 1950, 1952), however, that the spectrum of oxyhemocyanin shows marked similarities to those of certain cupric proteins. Attempts to resolve this conflict by magnetic and polarographic methods have not given unequivocal answers.

The status of the copper has now been established as follows. A dialyzed solution of hemocyanin, and one of biquinoline in glacial acetic acid, are deoxygenated by bubbling with purified nitrogen. The two solutions are then mixed under nitrogen. For oxyhemocyanin the same solutions are used, but they are kept exposed to air. When cuprous ion is present, a red copper-biquinoline complex is formed immediately; cupric ion gives no color. A typical set of results with plasma from *Busycon canaliculatum* is:

	Non-oxygenated	*Oxygenated*
Cuprous copper	$6.5 \times 10^{-4} M$	$2.9 \times 10^{-4} M$
Total copper	$7.9 \times 10^{-4} M$	$7.2 \times 10^{-4} M$

It is apparent first that copper is almost entirely in the cuprous state in the non-oxygenated protein. (The difference between total and cuprous copper may be contributed by biologically inactive protein.) Of greatest interest, however, is the observation that only approximately one-half of the cuprous copper is transformed to cupric during oxygenation. Since one molecule of oxygen is held by two copper atoms, it is clear that the active site consists of copper in each of two valence states. This information together with spectroscopic comparisons enables one to draw a structure for the active site which explains much of the behavior of hemocyanin.

Thus the oxygenation reaction in hemocyanin is markedly different in molecular nature from that in hemoglobin.

Controlled observation of hatching in Fundulus heteroclitus. ROGER MILKMAN.

In fish (except the grunion) hatching is normally far too unpredictable to observe satisfactorily. Controlled observation of Fundulus eggs has been made possible by specifically preventing hatching until desired. Normal development is otherwise unaffected.

Eggs are placed in about 100 cc. of sea water in a flask within a few days of fertilization, and oxygen is bubbled in. The flask is then stoppered until several days after the expected hatching date. Upon washing in sea water the eggs hatch in a few minutes (24° C.).

Although extra oxygen itself has an inhibitory effect on hatching, shaking oxygenated water removes the effect; but shaking does not remove the inhibition caused by the water the fish have been in, nor does temporarily making the pH 3 or 12, or heating to 65° C. Boiling removes the inhibition.

The hatching sequence begins with the simultaneous initiation of mouth activity, excretion of granular material from the anus, and contraction of the yolk sac to about ⅓ its former volume. Once this has occurred, hatching is assured, even though the eggs be replaced in inhibitor water. About 7½ minutes and 10 intrachorional somersaults later, the embryo breaks out.

During these 7½ minutes, the chorion changes from a smooth, tough, relatively thick coat, which keeps its shape, to a rough, weak, thin flaccid covering. The chorion originally has two layers, the outer being left as the thin, weak layer. The inner layer is quickly destroyed by an enzyme, but the fish must break out of the outer one. If a fish is anesthetized just before it is due to hatch, the thin layer remains intact indefinitely, although the enzyme retains its activity for days *in vitro*.

Because of its autonomy, its integration of rapid motor processes, and its result, the hatching process may be thought of as behavior.

The effect of oxygen on the frequency of x-ray induced mutations in Habrobracon sperm. W. E. Murphy.[1]

In the cross used in this experiment about 66% of the eggs are fertilized and diploid (female-producing); 34% are not fertilized and haploid (male-producing). When untreated females are mated to males x-rayed with sub-lethal doses, the number of sons remains unchanged while daughters are decreased due to dominant lethals induced in sperm. Each surviving daughter, bred unmated, demonstrates in her eggs (all unfertilized) her genotype in respect to recessive embryo lethals. If she carries none, all her eggs hatch, if one, 50% hatch, if two (non-linked), 25% etc. When heterozygous for a post-embryo lethal or a visible mutation, half her sons show it.

Two groups of males were x-rayed (4234 r), one in air, the other in nitrogen during irradiation, 100 seconds. Males exposed to nitrogen alone for twenty minutes bred as did controls, 96% hatchability of F_1 eggs, no mutations. Irradiated males were mated immediately to untreated females and then removed so that only sperm mature at time of exposure were tested. Hatchability of F_1 eggs was 38.4% for the air group and 45.5% for the nitrogen ($7.1 \pm \sigma_{\text{diff.}}$ 1.0), dominant lethal rate was 92.8% for former, 79.7% for latter ($13.1 \pm \sigma_{\text{diff.}}$ 0.03). Number of females produced per day per female was 5.6 ± 1.7 in controls, 0.9 ± 1.0 in nitrogen and $0.34 \pm .6$ in air. Recessive embryo lethals per F_1 female were 1.2 when sperm had been x-rayed in air and 0.55 when in nitrogen; recessive post-embryo lethals were carried by 7.7% of former, 8.6% of latter; visible mutations by 23.0% of former and 6.25% of latter. Tests of F_1 females are still being made. These differences when significant are smaller than those observed by Anna R. Whiting (unpublished) for eggs of Habrobracon irradiated under comparable conditions.

Adenosinetriphosphatase activity of spermatozoa. Leonard Nelson.[2]

$MgCl_2$ (10^{-4} M) increases the rate of ATP hydrolysis by whole Mytilus sperm which has been washed in filtered sea water (F.S.W.) and suspended in isotonic KCl to 220% of the control. All concentrations of $CaCl_2$ decrease the activity. If the sperm is washed twice in F.S.W., and then four times in isotonic KCl, Ca^{++} (10^{-3} M) increases the ATP-ase activity to 130% while Mg^{++} (5×10^{-4} M) raises the activity to 240% of the control. In combination the two cations antagonize each other.

The flagellar components of homogenized sperm have at least 13.5 times greater ATP-ase activity than the head fraction on a nitrogen content basis. Optimum ATP-ase activity in veronal buffer lies between pH 8 and 8.5. The temperature optimum occurs at approximately 30° C., and the activation energy of the crude tail suspension, over a range of 21°–27° C., is 13,700 Cal./degree/mole. When ATP concentration is limiting, only the terminal phosphate is liberated. The enzyme activity is proportional to the quantity of tail suspension present in the reaction mixture.

In some respects, this material resembles muscle ATP-ase. The tail fragments may be precipitated out of sea water or isotonic KCl by the addition of 5 to 20 volumes of ice-cold glass-distilled water. Amino acid composition of tail and muscle protein hydrolysates will be compared as well as electrophoretic and ultracentrifugation measurements on the purified preparations.

Where is the plasma membrane of the unfertilized egg of Arbacia punctulata? A. K. Parpart and P. C. Laris.

The literature contains frequent reference to a plasma membrane immediately inside the vitelline membrane of the unfertilized egg of *Arbacia punctulata*. In addition, the cortical granules first described by Harvey, 1911, have been described as embedded in a gel-cortex just inside the plasma membrane. There is also evidence that while this egg cell is permeable to ethylene glycol, it is only very slightly permeable to glycerol and relatively impermeable to erythritol, xylose, glucose and sucrose.

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[1] This work was done while working with Dr. Anna R. Whiting under a grant from the Phi Beta Psi Sorority.

[2] Partially supported by a grant from the Faculty Research Council of the University of Nebraska.

We have observed the cortical granules of the unfertilized Arbacia egg by means of the television microscope under a variety of experimental conditions. These granules swell and "explode" within a few seconds after these cells are exposed to isosmotic solutions of ethylene glycol, glycerol and erythritol and in 50% of eggs in xylose and glucose. Following the explosion of these granules a membrane is lifted from the egg in a manner analogous to the formation of the fertilization membrane including the marked churning of the cytoplasm immediately adjacent to the cortex. The remainder of the egg, however, does not increase in volume except in the case of ethylene glycol. Also these cortical granules cease to refract light when the egg is exposed to isosmotic sucrose though this can be reversed by replacing the sucrose with sea water. Such reversal can be repeated many times. The refractive index of this sucrose solution must be similar to that of the granules. The sucrose must penetrate the gel-cortex, but not the cortical granules.

It is therefore concluded that the plasma membrane of the mature unfertilized *Arbacia* egg must be at the inner margin of the gel-cortex. The cortical granules are outside the plasma membrane and they must lie in the gel-cortex between the vitelline membrane and the plasma membrane.

Electron microscopic investigation of bumble bee wing muscle. DELBERT E. PHILPOTT.

This investigation was carried out using material sectioned with a microtome designed by the author. The microtome consists of a motor-driven specimen holder which rotates in a vertical circle. The knife advance and holder are complete in one unit, thus simplifying the construction and operation. The coarse and fine mechanical advance is produced from one gear by dropping a worm gear (100:1 ratio) down on the coarse gear advance. This moves a rod whose opposite end holds the glass knife. The rod is electrically heated to produce the final advance of 2–300 Å per section. It has proved an economical and simple microtome to operate and construct. The bee wing muscle was fixed in neutral buffered osmic acid entering from the anterior end and fixing *in vitro*, thus preventing isotonic contraction. The tissue was then removed, dehydrated in isopropyl alcohol and embedded in methyl and butyl methacrylate (1:3) for sectioning. No cross band structure (A or I bands) has been seen either in longitudinal or cross sections. The protofibrils have been found to be packed in very regular hexagonal array. The diameter of one protofibril is 220 Å ± 15 Å and the mean distance from center to center of protofibrils is 350 Å, but disarrangement in fixation can vary this considerably. The sarcomer length is $2\,\mu$ and width of Z line is 800–1000 Å. An M line and thin H line also appear on all sections. The protofibril appears to get slightly narrower in front of the Z membrane and larger in it. The protofibrils are densely packed in an osmophilic substance in the Z membrane. The per cent shrinkage is estimated from 10 to 30%. This would increase the distance between protofibrils in the living state even beyond 130 Å. The Z band is tightly packed so probably there is but little shrinkage in it.

Cation antagonisms. D. R. SHANKLIN AND PHILIP B. ARMSTRONG.

The classical work of Loeb on salt antagonisms was done on *Fundulus* embryos. Believing that newer methods would facilitate studies of this kind, *Fundulus heteroclitus* embryos were subjected to varying concentrations and osmotic strengths of the chlorides of calcium, magnesium, potassium, and sodium. Exchanges of these cations across the ectoderm of Oppenheimer stages 22 to 27 were studied by radiotracers Na^{22}, Na^{24}, K^{42}, and Ca^{45}; magnesium was studied by a modified TCA titan yellow method for total Mg, corroborated by a method using 4-p-nitrophenylazoresorcinol, and exchangeable magnesium by a modified molybdivanadate method for phosphate; flame spectrophotometry was used for total sodium and potassium, and calcium was measured by a chelate titration of murexide complex with ethylenediaminetetracetate. That the site of salt-acid antagonism is the ectoderm and not the chorion was shown by Armstrong (1928) and by Armstrong and Shanklin (1952); thus salt antagonisms were studied at the ectoderm. These studies show that the salt effects are not all antagonistic. In several instances, *e.g.*, Na for K, and Mg for Ca, facilitation is profound. These results are tempered by knowledge of active transport mechanisms in *Fundulus* for influx and outflux (Shanklin, 1954). In osmotically balanced media, magnesium uptake is greater in all cases than is predictable from summation of unit action of the other ions on magnesium, while for Ca, K, and Na, the uptake is less than predictable. These results are in line with active mechanisms yielding a gradient inward for Mg, and outward for the others. The orders of action do not follow Hofmeister's lyotropic series, but appear to be specific properties of ions. The activity of ions in this system is Ca > K > Mg > Na, which is inverse to their concentration, and in the order of their atomic mass.

Cell movement and the axial gradient in Tubularia. MALCOLM S. STEINBERG.

The axial gradient in Tubularia is expressed by a decreasing rate of hydranth regeneration as one proceeds proximad down the stem. During regeneration there is a period of tissue movement toward the regeneration site, followed by the differentiation of a hydranth there. These experiments test the hypothesis that all differences in regenerative rate lie in the movement phase. In the first experiment 5-mm. sections were cut from stems, the distal cut being made 2 mm. below the base of the hydranth. Only sections of the widest and narrowest diameter were used, 20 wide pieces constituting one group and 20 narrow ones constituting the other. Time for regeneration was 48.5 hours for the narrow group and 59.1 hours for the wide group. Time for tissue movement was 22.5 hours for the narrow group and 32.7 hours for the wide group. Time for differentiation was 26.0 hours for the narrow and 26.4 hours for the wide group. In the second experiment twenty 10-mm. sections were cut similarly and ligated in the middle. Time for regeneration was 33.3 hours distally and 46.5 hours proximally. Time for movement was 21.4 hours distally and 33.0 hours proximally. Time for differentiation was 11.9 hours distally and 13.5 hours proximally. Repetition of this experiment on another colony gave 41.3 hours for regeneration distally and 75.2 hours proximally. Time for movement was 25.8 hours distally and 59.9 hours proximally. Time for differentiation was 15.4 hours distally and 15.3 hours proximally. The results show that while stems from different colonies differentiate at different rates, variations in regenerative rate between individual stems within the colony or between different regions of the same stem are due to differences in the rate of tissue movement and not in the rate of differentiation. Other experiments show that the movement is due to amoeboid ectoderm cells, which indicates that the axial gradient in Tubularia is in reality a reflection of the activity or orientation of these cells.

Sedimentation constants, viscosity, and diffusion of the fertilizins of Arbacia and Echinarachnius.[1] ALBERT TYLER, ALAN BURBANK AND JAMES S. TYLER.

Solutions of the fertilizins of *A. punctulata* and *E. parma* were prepared by the previously described (Tyler, 1948, 1949) methods. Sedimentation velocities were determined in the Spinco analytical ultracentrifuge, using relatively dilute (0.1% and 0.2%) solutions in pH 9.02 borate buffer ($\mu = 0.1$) forArbacia and pH 7.02 phosphate buffer ($\mu = 0.1$) for Echinarachnius. These gave average s_{20}'s of 7.09×10^{-13} cm./sec. unit field for Arbacia and of 4.67×10^{-13} for Echinarachnius, as compared with a previously (1949) obtained value of 6.3×10^{-13} for *Strongylocentrotus purpuratus.*

The intrinsic viscosities are 0.942 for Arbacia and 0.895 for Echinarachnius. Calculated as spheres, the molecular weights would be 82,800 for Arbacia and 54,000 for Echinarachnius.

Attempts were made to determine the diffusion coefficients by use of spreading of refractive index boundaries both in electrophoretic cells and in the ultracentrifuge (at low speeds). Since solutions of high concentrations gelate, the measurements were attempted with the relatively small areas and peaks obtained with dilute solutions, and these gave variations of over threefold. Taking as a first approximation 2.1×10^{-7} cm.^{2}/sec. for Arbacia gives a molecular weight of 300,000, and an axial ratio (as unhydrated prolate ellipsoid) of 28 : 1.

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[1] Supported in part by a grant (C-2302) from the National Cancer Institute, Public Health Service.

The electrophoretic mobilities of the fertilizins of Arbacia and Echinarachnius.[1] Albert Tyler, Alan Burbank and James S. Tyler.

Electrophoretic mobilities of the fertilizins of *A. punctulata* and *E. parma* were determined in the Tiselius apparatus in dilute (0.2%) solutions. The values obtained (in $cm.^2/sec./volt \times 10^4$) are, for Arbacia, 1.99 in pH 7.02 phosphate buffer ($\mu = 0.1$), 1.57 in pH 4.84 acetate buffer ($\mu = 0.1$) and 2.02 in pH 2.0 HCl-KCl buffer ($\mu = 0.05$), and, for Echinarachnius, 1.82 in pH 7.02 phosphate buffer ($\mu = 0.1$). The value for Arbacia at pH 2 seems anomalous. The other values are of the same order as those previously (1949) obtained for Strongylocentrotus and show the fertilizins to be highly acidic glycoproteins of low isoelectric point.

Studies on urocanylcholine. V. P. Whittaker and I. A. Michaelson.

Urocanylcholine, first discovered by Erspamer and coworkers in certain Mediterranean molluscs, notably *Murex brandaris,* has now been identified spectroscopically in two related North Atlantic species, *Urosalpinx cinerea* and *Thais lapillus.* The active material was extracted from the whole organisms by the usual trichloracetic acid procedure. This extract contained much material with a non-specific U.V. absorption. Accordingly an amount of extract equivalent to about one gm. of tissue was poured onto a weak acid ion exchange resin previously buffered to pH 4.3 and eluted with 0.1 *M* NaH_2PO_4 followed by 0.1 N HCl. The U.V. absorption and biological activity on the frog rectus preparation of successive fractions was determined. During the elution with phosphate, a biologically inactive component with intense U.V. absorption at 270 mμ made its appearance in the early fractions; this was tentatively identified as homarine on the basis of (a) the wave-length of the absorption peak, (b) the invariance of the peak with pH. The next material to appear was acetylcholine, identified by differential assay on the frog rectus and clam heart and by its similar behavior on the column to synthetic acetylcholine. On elution with acid the pH of the effluent fell, and a component with strong U.V. absorption and biological activity made its appearance. The spectrum of this compound was identical in the range 240–350 mμ with Erspamer's curve for urocanylcholine; on acid or alkaline hydrolysis, activity was lost and the spectrum was replaced by that of urocanic acid. Urocanylcholine accounted for 80–90% of the acetylcholine-like activity of the original extracts and its concentration in *Thais* and *Urosalpinx* was 360 and 230 μgm./gm. fresh tissue, respectively.

PAPERS READ BY TITLE

Fluctuation in the form of the daily rhythm of O_2-consumption in fiddler crabs.[2] M. F. Bennett, F. A. Brown, Jr. and H. M. Webb.

The O_2-consumption of two species of fiddler crabs, *Uca pugnax* and *Uca pugilator,* was measured continuously for about 45 days from June 23 to August 5 under constant conditions of temperature and illumination. The temperature was 19.5° C. and the illumination was less than 1 ft. c. When the form of the persistent daily cycle of O_2-consumption was determined for each of several semilunar periods it was found that the form of these exhibited a cycle of change through the month. During the two semilunar periods commencing with the first quarter of the moon, the rate was generally high through most of the day with maxima about 3 A.M., 9 A.M., and 3 P.M., and with a predominant minimum about 8 to 10 P.M. For the period commencing at new moon, the three maxima were more clearly evident. For the periods beginning at the first quarter and full moon, the 3 P.M. maximum became progressively more dominant with a lower rate of O_2-consumption during the morning hours. In every period the lowest rates of the day occurred during the evening hours. These observations strongly suggest that the crabs possess a cycle of O_2-utilization about a synodic month in length.

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[1] Supported in part by a grant (C-2302) from the National Cancer Institute, Public Health Service.

[2] This work was supported in part by contracts NONR-09703 and NONR 122803 with the Office of Naval Research.

Some physiological properties of frog sperm.[1] G. S. BERNSTEIN.

Sperm of *Rana pipiens* and *R. clamitans* were obtained from extirpated testes mashed in buffered (3×10^{-4} *M* phosphate, pH = 7.00), 8.7% (v/v) Ringer's solution. Large pieces of testicular tissue were removed by filtration through cheese cloth. The motility of washed and unwashed sperm from both species was reversibly inhibited 10 to 20 minutes after being placed in an oxygen-free (alkaline pyrogallol) chamber. HCN (10^{-3} and 10^{-2} *M*) reversibly inhibited motility within 6 minutes. Added glucose did not prevent the inhibition of motility by lack of oxygen or HCN. The sperm were treated with inhibitors in further attempts to characterize their metabolism. Fluoride, iodoacetate, azide, and mixtures of these substances (all at 10^{-3} *M*) did not affect motility in three hours. The failure of these substances to inhibit motility may be due to their high degree of dissociation, and hence their low penetration into the sperm, at pH 7.00.

There are several available substances which may be used as substrates by frog sperm. *R. clamitans* sperm contain 1.2×10^{-7} γ of lipid phosphorus per cell. Unwashed suspensions of *R. clamitans* sperm contain 10 to 25 γ of reducing sugar per ml. Paper-chromatographic analyses showed that only one reducing sugar is present; this is an aldohexose (reddish-brown color with *p*-anisidine HCl) having an R_f value identical with that of glucose.

These data indicate that frog sperm obtain energy for motility from aerobic metabolism, possibly involving the use of phospholipid or glucose.

The response of fibrillar flight muscle to rapid release and stretch. E. G. BOETTIGER AND E. FURSHPAN.

Wing movements in some insects are produced by antagonistic fibrillar muscles, both stimulated in flight at frequencies that produce tetanus in the isolated muscles. We previously suggested that the speed of shortening releases the tension which does not reappear during the subsequent stretch. To test this theory, a preparation of the longitudinal flight muscle of the bumble bee was used.

This muscle tetanizes at 40 stimuli per second; produces up to 40 grams tension; shows very strong summation; contraction takes about 250 msec. and relaxation twice as long. A muscle 5–6 mm. long can shorten 0.3 mm. from *in situ* length. Stretching above this length 0.1–0.2 mm. increases active tension. Probable shortening during normal flight is 0.1 mm. A fast release of 0.1 mm. produces almost complete loss of tension. The tension returns to that characteristic of the new length in the usual manner.

When a passively stretched unstimulated muscle is stretched following a release, the tension rises in phase with the stretch. A stimulated muscle can be stretched after release with the return of only 40–50 per cent of the original tension. The remaining tension returns following the attainment of full length. This property does not depend on the time after release as much as on the stretch itself.

Persistent rhythms of O_2-consumption in carrots and potatoes.[2] F. A. BROWN, JR., R. O. FREELAND AND M. F. BENNETT.

Sections of carrots and pieces of potatoes bearing eyes, each weighing about five grams, were investigated continuously for a synodic month and hourly values of O_2-consumption were obtained. Under the conditions of constant temperature and constant very low illumination there were continuous variations in the rate of O_2-uptake but there was no overt similarity of the patterns from day to day. When, however, the data for the whole lunar period were examined for the existence of a rhythmic component of primary solar frequency, an unequivocal daily cycle was apparent for each plant. The carrots exhibited a principal maximum about 5 A.M. and a principal minimum about noon; the potatoes showed two conspicuous maxima, one at 5 A.M., and the other at 3 P.M., with a principal minimum about midnight. An analysis of the data to demonstrate any persisting primary lunar rhythm showed the potatoes to have a minimum at lunar zenith, followed by a principal maximum eight hours later and a lesser minimum at lunar nadir followed by a lesser maximum about four hours later. For the carrots the lunar cycle was tetramodal with the principal maxima following lunar zenith and nadir by nine and five hours, respectively. The presence of a lunar cycle of O_2-consumption was also apparent in the demonstration that for both plants the diurnal cycle calculated for the semilunar period, May 12–26 gave the major maximum in the A.M., and for the period May 27–June 9, in the P.M.

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[1] Aided by grants from the Lalor Foundation, the Research Committee of the Faculty of the University of Delaware, and by a contract (Nonr 160–015) between the University (Dr. R. R. Ronkin) and the Department of the Navy, Office of Naval Research.

[2] This work was supported in part by contracts NONR-09703 and NONR 122803 with the Office of Naval Research.

The primary lunar rhythm of O_2-consumption in the fiddler crab.[1] F. A. Brown, Jr., M. I. Sandeen and C. L. Ralph

The O_2-consumption of two species of fiddler crabs was determined continuously for more than forty days and the data analyzed to learn the form and phase relationships of any persistent primary lunar or tidal rhythm which might exist. The data, for analysis, were subdivided into five semilunar periods, each beginning with the day of one of the quarters of the moon. In both species, for the first semilunar period, there were found simple sinusoidal rhythms with maxima of O_2-consumption at the times of low tide in the habitat from which the crabs had been collected. In the period covering the second and third weeks, the amplitudes of the lunar cycles were greatly reduced with maxima not only at the times of low tide but also with new maxima appearing about halfway between. For the remaining three periods freshly collected *Uca pugnax* were added from time to time; the lunar cycles tended to remain more or less bimodal. For the *Uca pugilator* the third fifteen-day period of analysis, utilizing some of the initial lot of animals and some which had been freshly collected, had bimodal lunar cycles. During the succeeding fifteen-day period continuing with the same collection of crabs the cycles continued to be bimodal, but by the last semilunar period had become again unimodal but now with minimum rate of O_2-consumption occurring about the time of lunar zenith and nadir. It appeared, therefore, that the crabs kept in the laboratory gradually shifted the phases of their tidal rhythm from a minimum about the time of high tide, to the time of lunar zenith and nadir which in this instance was six hours out of phase with the habitat high tide.

Solar and lunar rhythms of O_2-consumption in the seaweed, Fucus.[1] F. A. Brown, Jr., M. I. Sandeen and H. M. Webb.

Tips of thalli of the brown alga, Fucus, aggregating about five grams, were placed in each of seven transparent respirometer vessels suspended in a black-lined constant-temperature bath provided with a constant illumination of 1 ft. c. at the water surface, and the O_2-consumption measured continuously over a period of more than 15 days. Fresh plant material was substituted every four to six days. The rate of O_2-consumption fluctuated greatly during the period of the study. The over-all average was about 49 ml./Kg./hr. but the rate during the period varied as widely as from about 30 to about 100 ml./Kg./hr. When the data were analyzed for the presence of a rhythmic component of primary solar frequency between the dates July 29, the day of a new moon, and August 12, about the time of a full moon, there was found to be a daily cycle which was essentially bimodal. There were two nearly equal maxima, one about midnight and the other about noon. Minima were seen at 6 A.M. and about 3 P.M. From a minimum to a maximum involved about a 10% increase in rate. When the primary solar rhythm was rendered random and the data analyzed for any rhythmic component of primary lunar or tidal frequency, there was found present a low amplitude primary lunar rhythm during this same period. The lunar rhythm was one in which there were broad maxima separated by minima occurring at the times of lunar zenith and nadir. From maxima to minima in the lunar cycle involved an increase of only about 6% in rate of O_2-utilization.

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[1] This work was supported in part by contracts NONR-09703 and NONR 122803 with the Office of Naval Research.

Osmotic resistance of dogfish and tautog erythrocytes. David Chalfin.

Osmotic resistance measurements were made of the erythrocytes of dogfish and tautog at two pH's, 5.8 and 7.5. The isotonic solution for dogfish is 0.35 *M* NaCl and for the tautog is 0.21 *M* NaCl. The fragility curves for the dogfish cells are different from those of the tautog and in each case the curves at pH 5.8 are different from those at 7.5. In tautog, the cells appear less osmotically resistant at pH 5.8 than at pH 7.5. The tautog hemoglobin is precipitated at pH 5.8 but not at pH 7.5 so that the amount of measured hemoglobin at the acid pH never approaches the amount in solution at the alkaline pH. In addition, however, the amount of hemoglobin in the hemolysates of presumably completely hemolyzed cells varied slightly with salt concentration at pH 5.8 but not at 7.5.

In dogfish the cells appear slightly more osmotically resistant at pH 5.8 than at pH 7.5. This hemoglobin, in contrast with that of the tautog, is not precipitated at the acid pH. The shape of the osmotic resistance curve at pH 7.5 has a bimodal character; *i.e.*, the dogfish cells are grouped into two main classes of either size, membrane resistance or some other governing factor. At pH 5.8 the amount of hemoglobin in the hemolysate goes through a maximum as the tonicity is increased from distilled water towards isotonicity. At this maximum (ca. 30% of isotonicity) the amount of hemoglobin measured is almost equal to that found in basic solutions of similar tonicities. This resulted in what appeared to be less hemolysis in distilled H_2O than in 5% of isotonicity, less at 5% than at 10% of isotonicity and so on up to the maximum at 30% of isotonicity (0.105 *M* NaCl). Further study indicated that this was due to the fact that the hemoglobin which had come out of the cells adhered to the ghosts at pH 5.8 and the amount of this adhesion was dependent on the ionic strength of the hemolysate.

Mitotic inhibition by caffeine and x-rays. Ralph Holt Cheney and Roberts Rugh.

Comparative effects of different caffeine molarities upon the development of the egg of *Arbacia punctulata* were stated in 1948 by Cheney. In 1940, Henshaw reported effects of x-ray irradiation on Arbacia as being most severe in the prophase stage. The current experiments submitted unfertilized eggs in filtered sea-water (SW) to x-rays at the rate of 6,000 roentgen units per minute for intervals totalling 1,000 r to 300,000 r; and/or pretreatment of 30 minutes in various caffeine molarities, prior to insemination with non-treated sperm in SW. The fertilization process and developmental rate were observed continuously for two hours and at intervals to pluteus larva. Study was made of the effect of a given amount of irradiation and of a given caffeine molarity, separately and in combination. The chronology and extent of delay were recorded in the different mitotic phases of the first cleavage. Any over-all time delays in subsequent development were noted with reference to the organism's attainment of pluteus formation.

In agreement with Henshaw, the greatest effect (slowing up) by x-ray irradiation was observed during prophase. Our observations demonstrate clearly that the chemical agent, caffeine, is also most effectively inhibitory during amphiaster transitions of mitotic prophase. Caffeine and x-rays appear to affect mitosis at the same stage, *i.e.*, prophase. Therefore, we considered the possibility of synergistic, summation, or even antagonistic action of these two agents. Since no such effects were observed when these agents were applied in combination, it is presumed that the mechanism of their effect is different. Since it is known that x-rays alter the physical make-up of chromosomes and that caffeine affects the cortical granules and surface forces of the egg, we may suggest that x-rays delay cleavage by their effect on chromosomes and the first effect of the caffeine is primarily on the cytoplasm.

Uptake of certain hexoses by Arbacia larvae. G. H. A. Clowes, A. K. Keltch and C. P. Walters.

To supplement previous studies by the authors upon hexokinase activity of unfertilized and fertilized Arbacia eggs, hexose uptake of Arbacia larvae has been measured. Fertilized eggs were allowed to develop in sea water at 25° C. for 24 hours with continuous gentle stirring, yielding a homogeneous population of plutei; these were collected by gentle centrifuging, resuspended in sea water containing the desired hexose at .0004 *M*, and incubated for two hours at 20° C. in Warburg vessels with shaking at 70 cycles per minute. Glucose was taken up at an average rate of approximately 250 micrograms per hour per gram wet weight of larvae; this rate was not affected by dinitrocresol at concentrations from 4×10^{-6} *M* to 1.0×10^{-3} *M*. The use of mannose and galactose was small as compared to glucose; fructose did not appear to be used at all; exact values for hexoses other than glucose cannot be stated because the Nelson method is not completely satisfactory for these sugars. When the hexose added was fructose, there was a net increase in reducing substances during incubation. The oxygen consumption of the larvae, as in the case of unfertilized and recently fertilized eggs, rose to a maximum at 8×10^{-6} to 1.6×10^{-5} *M* dinitrocresol and decreased at higher concentrations; the addition of any hexose to the medium did not alter the basal oxygen consumption or the form and height of the curve with dinitrocresol.

Retention of phosphate label by starving Amoeba proteus. ADOLPH I. COHEN.

Amoeba proteus were indirectly labelled with P^{32} via feeding them *Tetrahymena pyriformis* which had been grown in media containing inorganic P^{32}. Single amoebae may be labelled to the extent of 500–1000 counts per minute after 48 hours of growth on such labelled ciliates. After growth on unlabelled ciliates for 48 hours to obviate label loss by defecation, single amoebae in a standard fluid volume and in a standard container were assayed daily, while living, for radioactivity. Such measurements were made over 10 days of starvation in various media or during feeding. Feeding amoebae divide and the total resulting population is then counted simultaneously as a unit. Starving amoebae usually show one division (presumably from reserves) and occasionally a second division, even after visible size loss and loss of reduced weight. In confirmation of the work of Frederich-Freksa, no loss of activity was found for the fed, growing population. Surprisingly, no detectable loss of activity was likewise found for the starving group. The amoeba medium employed has 8 mg. KCl, 4 mg. $MgSO_4 \cdot 7H_2O$, and 12 mg. $CaHPO_4 \cdot 2H_2O$ (saturated) per two liters of Pyrex-distilled water. The omission of phosphate ion, the addition of glucose (.004 *M*), the addition of sodium glycerophosphate (.004 *M*), and various combinations of these did not alter the findings. The method appears capable of detecting losses of over 15%. The loss of reduced weight during the starving period is of the order of 50%. Since James has reported loss of ribonucleic acid during starvation of whole amoebae, an attempt was made to fractionate amoebae and look for shifts in label distribution. The attempt to get a reliable fractionation procedure has thus far been unsuccessful and efforts along this line are continuing.

The effect of tetrodotoxin and related substances on cell division.[1] PIERRE COUILLARD.

It was suggested by Yudkin in 1945 that tetrodotoxin, the poison from the ripe ovary of tropical tetrodont fishes, can also occur in *Spheroides maculatus,* the common "puffer" of the North-Atlantic coast.

Ripe ovaries of *S. maculatus* contain a dialyzable factor which is active in preventing germinal vesicle breakdown in the oocyte of Asterias and cleavage in the eggs of Asterias, Chaetopterus and Arbacia. Like tetrodotoxin (which is also dialyzable), this factor is lacking in immature or spent ovaries. Crystalline tetrodotoxin (kindly supplied by the Sankyo Co., Tokyo) was shown likewise to inhibit both germinal vesicle breakdown and cleavage.

Spent ovaries do contain an antimitotic substance, but it is not readily dialyzable. Furthermore, crude extracts of such ovaries will markedly enhance germinal vesicle breakdown in starfish oocytes. In all cases the effects are not due to changes of pH.

Previous work, notably experiments with inhibitors such as periodate, points to this non-dialyzable antimitotic agent as being a mucopolysaccharide, having a heparin-like effect on blood clotting, possibly homologous with a number of such substances found by Heilbrunn and his co-workers in the ovaries of many vertebrate and invertebrate forms including 12 local species of fishes. Protein-free polysaccharides, containing from 5 to 8% glucosamine, have indeed been extracted in crude form from puffer ovary. Though they are metachromatic and occasionally exhibit anticoagulant action, they have failed, so far, to show antimitotic activity. This could be due to the lack of an essential protein cofactor (as recently shown by Rathgreb and Sylvén in the case of Shear's polysaccharide).

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[1] Holder of a Special Scholarship, National Research Council of Canada. Part of this investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

A conjugating strain of Tetrahymena vorax.[1] ALFRED M. ELLIOTT AND ROBERT E. HAYES.

Two clones of *Tetrahymena vorax* were isolated in April, 1954, from a small roadside stream near the village of La Chorrera in the Republic of Panama. Species identification was made from Feulgen and silver nitrate preparations following the taxonomic system proposed by Corliss. The two clones are considered identical and are here designated as strain RP. They are maintained in axenic culture.

All animals possess a typical macronucleus and usually one micronucleus, or rarely two. No amicronucleate individuals have been observed. It appears to have a higher number of chromosomes, of approximately the same size, than the conjugating strains of *T. pyriformis,* WH6 and WH14.

Strain RP exhibits the polymorphic life cycle characteristic of the species. Transformation from a pyriform microstome stage to the cannibalistic macrostome form occurs readily in starved cell suspensions.

Animals washed in distilled water and allowed to starve for several hours conjugate (self) within the clone. The stages of conjugation are essentially like those of *T. pyriformis.* At the end of the process conjugates separate and give rise to viable exconjugant clones, which are sexually immature.

Tetrahymena from Mexico, Panama, and Colombia.[1] ALFRED M. ELLIOTT AND ROBERT E. HAYES.

One hundred forty-two fresh-water samples from Mexico (37), Panama and the Canal Zone (81), and Colombia (24) were examined for *Tetrahymena.* The habitats included rivers, ponds, lakes and irrigation ditches. They ranged in elevation from sea level to 10,000 feet, and in temperature from approximately 8° to 35° C. Of these 142 samples 30 were positive, and from these 277 clones of *Tetrahymena* were isolated and established in axenic cultures. Two proved to be *T. vorax;* all others, *T. pyriformis.*

None of the 277 clones were able to grow when any one of the 19 nutrilites essential for strain E were absent from the medium. Their growth characteristics in the standard peptone-tryptone stock medium resemble those of strain E.

The nuclear picture varied among the different clones. For example, from two habitats in Mexico and 10 in Panama, all contained micronuclei, whereas 5 other Mexican and 5 Panamanian habitats yielded only 11 out of 41 clones that contained micronuclei.

All clones were tested for their mating reaction. Conjugating strains were found in 12 of the 30 habitats studied, all of which belong to variety 2 of Gruchy's mating system for *Tetrahymena pyriformis* (except the *T. vorax* strains, which are selfers). Conjugating strains were also found from 5 additional habitats but they have not been identified as to variety. All mating strains possess micronuclei. No amicronucleate clones have been observed to conjugate.

X-irradiation of sexual strains of Tetrahymena.[1] ALFRED M. ELLIOTT, ROBERT E. HAYES AND J. ROGERS BYRD.

The non-conjugating, amicronucleate strain E (*T. pyriformis*) is highly resistant to x-rays. The mating types, WH6 and WH14, on the other hand, do possess micronuclei and should furnish more favorable material for detecting radiation damage to the genetic system.

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[1] This investigation was supported in part by a research grant (PHS G3588) from the National Institutes of Health, Public Health Service.

Distilled-water suspensions of vegetative cells of both mating types were exposed to graded doses of x-rays employing the Wichterman-Figge plastic radiation chamber. At 50,000 r intervals samples were removed and placed in peptone-tryptone stock culture media. Following 72 hours of incubation the amount of growth was determined. No animals survived 650,000 r and the LD 50 was computed to be approximately 400,000 r. Surviving cells which received 200,000 to 600,000 r, inclusive, were permitted to conjugate and a search was made for evidences of cytological abnormalities. The conspicuous anomalies were failures of the homologous chromosomes to pair properly.

In order to determine the relative sensitivity to x-rays at various stages in the life cycle, mating types were first permitted to conjugate and reach the crescent stage of prophase of the first prezygotic division, and then exposed to graded doses of x-rays. Samples were withdrawn at 5000 r intervals. Twenty pairs were isolated from each sample and placed in stock media in depression slides. After 24 hours of incubation they were transferred to tubes. Ninety-six hours later relative growth was determined. No cells survived 400,000 r and the LD 50 was computed to be approximately 300,000 r. Cells that survived conjugation gave rise to viable cultures which appeared to be normal as regards their cultural reaction in stock medium.

Excitation in nerve cells of the lobster stretch receptors. C. EYZAGUIRRE AND S. W. KUFFLER.

The stretch receptors in the lobster (*Homarus americanus*), recently described by Alexandrowicz, offer a suitable preparation for the study of many aspects of nervous activity. Each stretch receptor unit consists of a fine muscle bundle which receives one sensory neuron and one or more motor and inhibitory nerves. The sensory discharge of stretch receptors is controlled by passive stretch, by motor nerve impulses activating the muscle elements of the receptor and by nerve inhibitory action. The cell body of the sensory neuron, situated in the periphery, sends dendrites into the muscle elements. Contraction or stretch of the receptor muscle causes excitation of the dendrites. By recording with intracellular electrodes from the nerve cell many events leading up to excitation were analyzed. By stretch deformation the dendrites become depolarized and thus they serve as generators for the subsequent events in the nerve cell. The generator potential within the dendrites is transmitted electrotonically to the adjoining cell body which fires propagated nerve impulses at a critical level of its membrane potential. The dendrite potentials are graded dependent on the extent of stretch and accordingly they "drive" the discharge in the adjacent cell body at different rates. Some receptor neurons adapt quickly and thus serve to register transient tension changes, while others adapt slowly and discharge for the entire duration of stretch. It is noteworthy, however, that in both neurons the level of the membrane potential in the cell body of the sensory neuron reflects faithfully the changes in the "generator" located in the nearby dendrites. Thus the level of the cell membrane potential can be "set" for long periods and thereby the excitability of the cell is adjusted. The dendrite-nerve cell system resembles an electrical transducer and by recording the membrane potentials, minute contractile changes in the receptor have been detected.

Inhibitory activity in single cell synapses. C. EYZAGUIRRE AND S. W. KUFFLER.

The sensory neuron of the lobster stretch receptor makes contact with the contractile receptor elements through dendritic processes. These dendrites also carry synapses from "accessory" fibers (Alexandrowicz). These fibers have now been identified as inhibitory and some of the mechanisms of inhibition have been studied. The action of inhibitory synapses directly affects the "generator" potential in the dendrites and thus indirectly controls the excitability of the adjacent cell body and the discharges of the sensory neuron. Inhibitory nerve impulses cause potential changes which depend on the level of the membrane potentials (M.P.). If the M.P. level is reduced by a constant stretch, inhibitory nerve impulses will repolarize it; in the absence of stretch with the M.P. near its "resting" state, inhibition may cause no detectable hyperpolarization, but may even set up a small depolarization. The main inhibitory action, therefore, appears to consist in driving the membrane potential of a cell towards its "equilibrium" level. The effect of each inhibitory impulse, reflected in the membrane change, rises to its peak in a few msec. and decays in about 30 msec. Inhibitory activity can stop the sensory discharges in spite of strong stretch on the receptor, by first repolarizing the cell membrane and then "holding" or "stabilizing" it, thus preventing depolarization and excitation. The antidromic spread of a nerve impulse into the nerve cell may also be prevented by inhibition, but it is thought that this occurs only when the safety margin of propagation near the axon-soma boundary is low. These results are closely related to those of Katz and Fatt on crustacean nerve-muscle junctions and to those of Eccles and collaborators on the cat's spinal motor neuron.

Parasites in starved guts in well-nourished hosts. CHAUNCEY G. GOODCHILD.[1]

Relative dependence of gut parasites on food absorbed from the lumen as contrasted to food absorbed from the mucosa are unanswered questions of fundamental importance in parasitology. The rationale and operative procedure of the preliminary technique herein reported had been perfected before an identical procedure used in hormone studies, the so-called Thiry-Vella fistula, was discovered. Fourteen white rats, under ether anesthesia and after 24 hours starvation, had 2-cm. incisions made through the integument and body wall of the lower right flank. The small intestine was pulled through the opening and an 8–15 cm. section, with mesentery intact, was cut free. The intestine was then rejoined and the ends of the isolated section sewed at the ends of the incision. After 48 hours the animals were again given food and water. The U-shaped fistula was flushed periodically with mammalian Ringer's until healing had occurred in approximately 5–7 days. One or two tapeworms, *Hymenolepis diminuta*, from donor white rats, were then implanted, in mammalian Ringer's, deep into the fistula by means of a flexible polyethylene tube of proper bore. The parasites established themselves and survived in some hosts without exogenous food for 3 days. This technique, for the first time, permits placing gut parasites in starved guts, but in well-nourished hosts. It should also permit sterilizing the fistula with suitable antibiotics and administration of selected metabolites to determine relative dependence of many gut helminths, without microbial contaminants, on absorption from lumen as contrasted to absorption directly from the mucosa of the host.

The effect of insulin on the respiration of the sponge, Microciona prolifera. E. E. GORDON AND MELVIN SPIEGEL.

It was shown by Krebs and Eggleston (1938), using a pigeon liver suspension, that insulin could effect an increase in O_2 uptake. We have been able to demonstrate this action of insulin on an invertebrate—the sponge, *Microciona prolifera.*

O_2 consumption was measured by the conventional Warburg technique. Apical portions of sponge were cut into 2–5 mm. pieces, allowed to dry for approximately two minutes on absorbent paper and weighed. Either K pyruvate (0.001 M), glucose (0.0056 M), or Na_3 citrate (0.001 M) in sea water was used as substrate. Experiments were also performed without added substrate. Four units of regular insulin (Iletin, Lilly) containing 0.2% phenol as preservative were added to each of the appropriate vessels. As controls, distilled water or 0.2% phenol was added in place of insulin. The vessels were incubated for two or three hours at 37.2° C. in an air atmosphere. At the end of this period, aliquots of the medium were removed for chemical determination.

In the presence of insulin, O_2 uptake is increased an average of 16%. Controls utilized an average of 0.116 μl. O_2/mg. wet wt./hr. With added insulin the average O_2 uptake is increased to 0.134 μl./mg. wet wt./hr. Neither phenol, in concentrations present in the commercial insulin, nor any of the added substrates had an effect on O_2 utilization. No significant utilization of any added substrate by control and insulin-incubated preparations was detected. Determination of keto acids in the pyruvate-incubated preparations revealed a production of these acids.

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[1] This investigation was supported by a grant from the McCandless fund of Emory University.

The carbohydrate metabolism of the slime mold, Dictyostelium discoideum, during development.[1] James H. Gregg and Ruth D. Bronsweig.

The slime mold, *Dictyostelium discoideum*, derives its energy solely from an endogenous food supply during morphogenesis. During the transformation from the migrating pseudoplasmodia to the mature sorocarps certain nitrogenous components are utilized. It was of interest to investigate the role of the carbohydrates during morphogenesis, not only from the standpoint of determining the amount of energy-yielding substrate utilized but also to determine the source of the extracellular cellulose which is deposited around the stalk and spore cells of the mature sorocarp.

The dry weights of the slime mold amoebae, migrating pseudo-plasmodia, mature sorocarps, spores and stalks were determined by weighing on a quartz helical balance. The tissues were then analyzed for total reducing substances which were assumed to be primarily reducing carbohydrates. The total reducing substances/dry wt. show a consistent increase from the vegetative amoebae to the mature sorocarps. The stalk component of the mature sorocarps showed a greater increase in total reducing substances than the spores. The increases in total reducing substances/dry wt. may reflect a possible loss in dry weight which might occur during the development of the slime mold. However, it seems clear from the data that protein is the ultimate major substrate utilized for purposes of obtaining energy and satisfying other morphogenetic requests rather than the carbohydrates.

Electrical and mechanical manifestations of muscle activity caused by rapid cooling. Rita Guttman and Milton M. Gross.

The anterior byssus retractor muscle of *Mytilus edulis*, previously potentiated with potassium (from 39 to 91 millimols KCl added to sea water), was subjected to rapid cooling. The potential difference between one end of the muscle immersed in 0.26 *M* KCl and the other experimental end of the muscle was measured by means of a galvanometer used as a null point instrument. Virtually isometric contractions were recorded on a kymograph. Depolarization occurs on rapid cooling, the degree of depolarization depending upon the amount of cooling. Contractions occur whenever the cooling is rapid and the amount of depolarization exceeds a critical threshhold value. If the cooling is gradual, the amount of depolarization is about the same, but no contraction occurs. After the action potential has been initiated by rapid cooling (from about 22° C. to 2° C.), recovery occurs in about 80 minutes, if the cold temperature is maintained. This recovery consists of a return to a steady state with a P.D. about 50% of the original value.

Changes in the protoplasm during maturation.[2] L. V. Heilbrunn and Walter L. Wilson.

It has long been known that the protoplasm of eggs in the germinal vesicle stage is more viscous than it is after the germinal vesicle breaks down, and it has generally been assumed that the liquefaction of the protoplasm following germinal vesicle breakdown is due to the release of substances from this structure. In Chaetopterus eggs, germinal vesicle breakdown is induced by the entrance of the eggs into sea water. Goldstein in 1953 showed that as the eggs left the ovary and entered sea water, the protoplasm of the cortex became less rigid. Calcium is apparently released, and this calcium activates a proteolytic enzyme system. Such a protease might clot the protoplasm and it might also liquefy the protoplasm by dissolving some viscous constitutent of it. Actually, in the Chaetopterus egg it does both. The entrance of the egg into sea water is immediately followed by a sharp increase in protoplasmic viscosity. At 21°, the time relations are as follows. Within 30 seconds after the eggs enter sea water the viscosity doubles, but this increase is very transitory, for one minute after the eggs have entered the sea water the viscosity is approximately the same as in the ovarian egg. From then on the viscosity drops rapidly so that 5 minutes after the entrance of the eggs into sea water the viscosity is only ¼ as great as in the ovarian egg. The germinal vesicle does not break down until 7 minutes after the eggs have entered the sea water. Thus, apparently, a calcium-activated protease can cause both gelation and liquefaction of protoplasm, and the liquefaction does not depend on the breakdown of the germinal vesicle.

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[2] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

Some properties of the regenerating limb blastema cells of salamanders.[1] H. HOLTZER, G. AVERY AND S. HOLTZER.

Recent analysis (S. Holtzer, 1954) of the morphogenetic mechanisms in tail regeneration in salamanders suggested that: (1) the cells of the regeneration blastema form cartilage primarily, the muscle originating directly from the muscle of the stump, and (2) the regenerating spinal cord by patterning the mesodermal tissue is responsible for the morphology of the tail. The following experiments were designed to ascertain whether (a) cells of the salamander limb blastema likewise differentiated primarily into cartilage and (b) the mesodermal tissues of the limb were responsive to the patterning effects of the spinal cord.

Clusters of 3 or 4, 9-day limb blastemata were introduced into tunnels in the dorsal fin of a salamander host and allowed to remain for two weeks. The blastemata fused, grew and eventually formed large masses of organized cartilage. Long bones, metacarpals, phalanges and joints could be observed. No differentiated muscle fibers were present. Thus limb blastema cells grown under quasi-tissue culture conditions differentiated primarily into cartilage.

The following preparation served to test the reactivity of the limb mesoderm to the action of the spinal cord. The humerus was removed and a piece of spinal cord of similar length substituted. Following a two-week healing period, the limb was amputated through the graft. Though many bizzare structures regenerated, for the most part they were clearly limb-like. In addition to generalized enlargement of the regenerated tissue, often projections consisting of epidermis and a cell-sparse connective tissue formed. These were strikingly similar to fin tissue and probably attributable to the activity of residual neural crest derivatives included with the spinal cord. Failure to observe tail structures (*e.g.*, segmented muscles, vertebral cartilages, etc.) in these experiments suggests the spinal cord is incapable of: (1) modeling limb mesoderm in a fashion analogous to its action on trunk mesoderm, and (2) inducing vertebral cartilages from limb mesoderm.

The inductive role of the spinal cord in tail regeneration in the salamander.[1] SYBIL HOLTZER.

Though parallels between embryogenesis and regeneration have been pointed out, the phenomenon of induction has never been included in such a list. Analysis of tail regeneration in the salamander indicates that induction is truly a component of that process and can be shown to operate in a variety of experimental situations. In this particular system the spinal cord serves as inductor while the somitic mesoderm responds. The interactions between the two are as follows: In normal tail regeneration the notochord does not regenerate; rather, directly below the motor surface of the spinal cord, appear segmented cartilages, the centra, which later develop neural and haemal arches. That such cartilages are not derived from notochordal cells is shown by the fact that they may be obtained by (a) amputation of cord-muscle deplants into the dorsal fin, (b) grafting of nerve cord directly under the skin of flank and tail, (c) displacement of nerve cord to a more dorsal position in the midline. No centra are produced by amputated deplants of notochord or notochord and muscle in the dorsal fin.

It is to be emphasized that the centra form only along the motor surface of the spinal cord, regardless of the D-V orientation of the latter with respect to the muscle. Presumably the cartilage promoting activity resides exclusively in the motor half of the spinal cord.

Other results indicate that the muscle in the regenerated tail buds off from that in the stump, unilateral deficiencies there being preserved in the regenerate. Muscle will regenerate a limited amount in the absence of nerve cord but will form no cartilage. Contrary to previous reports tail and limb regeneration systems are comparable in that both can regenerate skeletal elements though such elements are absent in the stump.

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[1] Supported by a U.S.P.H. and a Woods Hole-O.N.R. grant.

Ecology of the intertidal fauna of the Woods Hole region. ROBERT STEARNS HOWARD.

At Gansett Beach in Woods Hole, Massachusetts, the amphipod *Orchestia* is the most abundant macroscopic organism at the low-tide mark. Its dark-brown color provides camouflage in the wet sand. Up-beach, the whitish *Talorchestia* replaces *Orchestia,* its color likewise offering camouflage in the dry sand. Along rocky or sandy shores, the green crab *Carcinides* is the dominant organism, being replaced in muddy areas by the mud crab *Neopanope.* Halacarid mites, nereid annelids, the gastropod *Littorina,* the barnacle *Balanus,* the pelecypods *Mytilus* (in rocky areas) and *Brachidontes* (in muddier areas) are abundant at the low-tide mark. These apparently require submersion most of the time. Up-beach (or higher on rocks in the same area), the barnacle *Balanus* (with porous plates) is replaced by *Chthamalus* (with non-porous plates). Between the high- and low-tide lines is a narrow band (often less than three feet wide) where pseudo-scorpions (*Pselaphochernes?*) occur. Here the collembolan *Anurida* (found also nearer to or walking on the water) is found in abundance under rocks. Trombidiid mites, pseudoscorpions, and the pselaphid beetle *Brachygluta luniger* feed on the collembolans here. The collembolans feed on detritus. Farther back (ca. 18 feet from the low-tide mark), only gammarid crustaceans are found. Four feet back (ca. 22 feet from the water), the seaside earwig *Anisolabis maritima* is found along with the gammarids. Four feet farther back (ca. 26 feet from the water), a few typically inland terrestrial forms appear, such as millipedes, centipedes, etc. The earwig *Anisolabis* is abundant from this region to the drift line, but is not found farther back.

Comparative ecology and behavior of two sympatric earwigs, Forficula auricularia Linnaeus and Anisolabis maritima (Gene). ROBERT STEARNS HOWARD.

Though sympatric, the European earwig *Forficula auricularia* Linnaeus and the seaside earwig *Anisolabis maritima* (Gene) are separated ecologically. *Anisolabis* is restricted to the intertidal zone; *Forficula* is never found there, though a distance of only ten feet may separate the two. *Anisolabis* lives among the pebbles and debris near the high-tide mark. *Forficula* is most abundant where thick sod abuts a solid surface. *Anisolabis* is highly cannibalistic even when food is abundant. *Forficula* may eat live insects (and even fight over them), but it displays no cannibalism. In *Forficula,* there are two morphological types of males, the "regular" type and the *forcipata* type with greatly enlarged forceps (over twice the size of the "regular" forceps); 317 (45%) of 702 males examined were of the *forcipata* type. Both types were found in all aggregations examined. Trios (one female, one "regular" male and one *forcipata* male) tested for dominance and subordination proved the *forcipata* dominant in 36 (64%) of 56 cases where dominance was demonstrated; 46 additional cases were inconclusive. In both species, the forceps are used in fighting, threatening, defense, courting and copulation. *Forficula* uses the forceps also to help fold the flying wings under the tegmina. *Anisolabis* is wingless. In *Anisolabis* the female always dominated the (somewhat smaller) male. In *Forficula,* the sexes are about equal in size, and neither sex dominated. The only regular domination displayed was of the *forcipata* type over the regular type of male.

A case of apparent physiological competition between ethylene glycol and glycerol. M. H. JACOBS.

Evidence has been given elsewhere that the entrance of glycerol into the erythrocytes of man and several other mammals takes place by two processes: (1) a relatively slow one of simple diffusion through widespread non-specialized pathways, as in other cells, and (2) a rapid one, apparently limited to small specialized portions of the surfaces of the erythrocytes in question. The rapid process can be reversibly inhibited by moderate acidity, traces of copper, and other agents that have little effect on the slower one. The 2-carbon homologue of glycerol, ethylene glycol, enters all cells so far studied with great ease, presumably by simple diffusion. Studies of permeability constants, however, have shown that in erythrocytes possessing the rapid mechanism for glycerol the rate of entrance of ethylene glycol also tends to be more rapid than in the case of other cells, and further that this "extra" rapidity, like that of glycerol, is sensitive to acidity and traces of copper. The following observations seem to suggest that ethylene glycol can successfully compete with glycerol for the "rapid" pathway. (1) In the presence of ethylene glycol the entrance of glycerol into human erythrocytes may be greatly slowed. (2) The same and even higher concentrations of ethylene glycol have little effect on the entrance of substances believed to follow unspecialized pathways (thiourea, diethylene glycol, triethylene glycol) or specialized pathways different from those used by glycerol (anions). (3) Ethylene glycol does not slow the entrance of glycerol into beef erythrocytes, which lack the fast process. (4) Ethylene glycol has little or no retarding effect on the entrance of glycerol by the slow process into human erythrocytes in which the fast one has been inhibited by acidity or copper.

Valence changes in the oxygenation of hemerythrin. I. M. Klotz, R. A. Resnik and T. A. Klotz.

By analogy with hemoglobin it is generally assumed that the iron in hemerythrin is in the ferrous state, both in the deoxygenated and oxygenated forms. This view has been strengthened by the observation (Marrian, 1927; Florkin, 1933; Roche, 1933) that oxidizing agents, which presumably transform the iron into the ferric state, convert hemerythrin into methemerythrin which no longer combines with oxygen. Since oxidizing agents give misleading results, however, as regards the state of copper in hemocyanin, some doubt is raised as to the significance of similar experiments with hemerythrin. Furthermore, the effects of oxidizing agents give no insight into the status of the metal in oxyhemerythrin.

The status of the iron has now been established as follows. A solution of crystalline hemerythrin in sea water and one of *ortho*-phenanthroline in glacial acetic acid are deoxygenated by bubbling with purified nitrogen. The two solutions are then mixed under nitrogen. For oxyhemerythrin the same solutions are used, but they are kept exposed to air. When ferrous ion is present, a pink-colored, iron-phenanthroline complex forms slowly; ferric ion produces no color at all.

With oxyhemerythrin, all of the iron is released in the ferric state. With deoxygenated hemerythrin almost all of the iron is in the ferrous state; approximately 25% may be in the ferric form. It is possible, however, that this small fraction of ferric ion is contributed by biologically inactive protein, for hemerythrin which has been allowed to stand at room temperature in air for several days, until it loses its capacity to combine with oxygen, shows only ferric ion even in the absence of oxygen.

It becomes apparent, therefore, that unlike hemoglobin, hemerythrin actually undergoes a change in the valence state of its iron in the oxygenation reaction.

Activity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in relation to glycolytic enzymes of Arbacia eggs. M. E. Krahl, A. K. Keltch, C. P. Walters and G. H. A. Clowes.

Glucose-6-phosphate (GP) and 6-phosphogluconate (PG) dehydrogenases of Arbacia eggs were determined from rates of triphosphopyridine nucleotide (TPN) reduction at 23° C. in the Beckman spectrophotometer at 340 mμ. Egg fractions were obtained as previously described. Final concentrations in the test system were: KCl, 0.08 M; glycylglycine, 0.025 M; $MgCl_2$, 0.03 M; TPN, 0.00005 M; egg protein, 0.1–1 mg.; substrate, as desired; total volume, 2–3 ml. Rates are average values for the first two minutes after substrate addition and refer to the extract from one gram eggs. At 0.001 M GP, the rate of TPN reduction by the homogenate of unfertilized or 30-minute fertilized eggs was 2.5–3.5 micromoles per minute, enough to support a rate of oxygen consumption 25 times that observed for unfertilized, and 6 times that for fertilized, eggs; 95 per cent of this activity was recovered in the supernatant fraction after centrifuging at 20,000 g for 30 minutes. With the supernatant fraction the concentrations of GP and PG for half maximal activity were each approximately 0.00004 M for the respective enzymes. Maximal activity with PG as substrate was 50–60 per cent of that with GP. During oxidation of GP by the supernatant fraction of unfertilized eggs the pentose formed was at a rate 0.3–0.5 that of TPN reduction. Rates of TPN reduction with other substrates at 0.001 M were: glucose-1-phosphate, 0.4; fructose-6-phosphate, 2.6; fructose-1,6-diphosphate, 0.8, indicating presence of phosphoglucomutase, isomerase, and a fructose-1,6-diphosphatase, respectively. Reduction of diphosphopyridine nucleotide in presence of fructose-1,6-diphosphate and adenosine diphosphate was 0.1–0.2 micromoles per minute, indicating that the glycolytic pathway over aldolase and oxidizing enzyme can metabolize GP at about 5 per cent the rate at which it can be oxidized by the TPN system derived from unfertilized or fertilized Arbacia eggs.

The effect of cortisone and of corticotropin (ACTH) on the rate of cell division.[1]

VALY MENKIN AND MAX PEPPER.

Earlier studies by one of us (V. M.) have indicated that previous to fertilization, exposure of *Arbacia punctulata* ova to cortisone and ACTH reduces the incidence of cell division. Cortisone acetate administered 30 to 40 minutes after fertilization delays the rate of cleavage. The delay seems proportional to the actual concentration of the corticosteroid used. The amounts employed varied from 0.5 mg. to 2.5 mg. per 10 cc. of sea water. There follows within the first hour and a half a delay in the rate of cleavage ranging from 20% to 41% in the appearance of the 3–4 (or over) blastomeric stage. When the corticosteroid was added 1–10 minutes following fertilization, the effect was even more marked, the delay being 70% in the series studied. The use of a soluble salt of sodium cortisone phosphate (courtesy of Dr. W. Umbreit, Merck Co.), several minutes after fertilization, in concentrations of 7–10 mg. likewise delayed the cleavage in 6 experiments to an average of 39%. On the following day the controls usually displayed free swimming plutei larvae, whereas the experimental ova, as a rule, manifested a definite retardation in the development of their plutei. Testosterone propionate (2.5–5 mg.) as a control steroid failed to induce any appreciable delay in cleavage, the average retardation being only 12.2%. ACTH (Armour) yielded the same type of results as cortisone. Doses of 4–10 mg. per 10 cc. of sea water delayed the rate to the 3–4 blastomere stage by an average of 63%. The delayed effect on the development of plutei with the use of ACTH was also observed, just as in the case of cortisone.

The effect of cortisone on cellular permeability.[1]

VALY MENKIN AND MAX PEPPER.

The decrease in capillary permeability in inflammation induced by cortisone is well known (Menkin, 1940, 1942). Previous investigators have reported no striking changes in the appearance of marine eggs exposed to cortisone. Is the rate of penetration of water in the ova of *Arbacia punctulata* decreased by transferring the eggs to hypotonic sea water? The diameter of spherical ova was determined by an ocular micrometer. The eggs were then transferred to 50% sea water. The diameter of the ova was then measured at rapid successive intervals for 20–25 minutes. In each experiment 40–50 ova were measured. The same procedure was repeated with the addition of one mg. of cortisone acetate (Merck) in 10 cc. of sea water. In one observation 2.5 mg. of the corticosteroid was utilized. Overcrowding of ova was avoided in order to bring the suspended cortisone in as close contact with the ova as possible. The preliminary period of exposure to the corticosteroid prior to transfer to 50% sea water ranged from 22 to over 45 minutes. The ova were studied at a magnification of 440 ×. The diameter of the ova exposed to cortisone *per se* tended usually to show a very slight increase in size. Subsequent to their transfer to 50% sea water there followed a decrease in the rate of expansion of the eggs. This, although not invariably, occurred in the first few minutes after transfer. The average decrease in the expansion of the eggs in 6 separate

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[1] Aided by a grant from the U. S. Public Health Service, and by a grant from Dr. A. Wander, S.A., Berne, Switzerland.

experiments with one mg. concentration of cortisone acetate was 34%. In the additional experiment involving 2.5 mg. of cortisone, the decrease was 48%. These data are interpreted as an indication that cortisone acetate decreases somewhat, but definitely and consistently, the permeability of sea urchin ova to water.

The adjuvant action of chelating agents in fertilizin agglutination of starfish sperm.[1] CHARLES B. METZ.

Starfish fertilizin ordinarily has no visible effect upon species sperm but in the presence of an "adjuvant" the fertilizin agglutinates the sperm in a specific and striking manner. Animal sera, hen's egg white, peptides and α amino acids were found to be effective adjuvants in earlier studies (Metz, 1945; Metz and Donovan, 1950). Although these agents appeared to act by exposing more receptors (antifertilizin) on the sperm surface, the mechanism of this action has remained obscure. However, it seems likely that the adjuvant effect involves metal binding for the above agents can bind metals. Furthermore, certain other metal binding and chelating agents have now been shown to have adjuvant action. Finally, the adjuvant action of chelating agents is inhibited by metal cations.

Chelating agents which show adjuvant action include Versene (approximately 0.01 M in sea water; tested on: *Asterias forbesii, Luidia clathrata, Patiria miniata*) and 8-hydroxyquinoline (saturated solution in sea water; *Asterias, Luidia*). However, citrate (0.5 M) proved to be ineffective. Other effective metal binding or precipitating agents are thioglycolate (0.05 M in sea water; *Luidia, Patiria*) and hydrogen sulfide (saturated solution in sea water; *Luidia*). The adjuvant action of Versene is inhibited by Ni^{++} (*Patiria*); the similar action of 8-hydroxyquinoline is inhibited by Co^{++} and Cu^{++} but not by Ca^{++} (0.5 M).

These observations in conjunction with the earlier studies suggest that most of the sperm surface receptors (antifertilizin) are blocked by metal cations and are unavailable for combination with fertilizin. In the presence of the appropriate metal binding agent (adjuvant) the metal cations are removed, thereby freeing the receptors for combination with fertilizin. Under appropriate conditions this combination results in agglutination. This view further implies that the sperm surface receptors have metal binding properties. Sulfhydryl groups, α amino acid or other chelating structures may be essential parts of these receptors.

Reversible clotting in Spirogyra. W. J. V. OSTERHOUT.

When cells of *Spirogyra* are placed in 0.1 per cent duponol solution (chiefly sodium lauryl sulfate) which kills the cell, the chlorophyll leaves the chloroplasts and a green solution fills the cell. The green color diffuses out when the cell is cut in water.

When the intact cells are transferred from the duponol to a dilute lugol solution ($KI + I_2$) the contents of the cell become dark and shrink to a stiff gel forming a cylindrical mass in the center surrounded by a colorless liquid. When the cell is cut in water the stiff gel comes out and maintains its form.

The shrinkage in the intact cell is not reversible when the cells are transferred from the lugol solution to water or to buffer solutions at various pH values but it is reversible when the cells are transferred back to 0.1 per cent duponol. The stiff gel disappears and the cell is again filled with the green solution.

This may be repeated many times.

If iodine is omitted the shrinkage occurs more slowly but it is reversible in duponol.

If cells are placed first in lugol solution and then in duponol there is little or no shrinkage.

Shrinkage occurs in lugol solution with cells that have been left standing in 0.1 per cent duponol from a few minutes to several weeks.

If cells are first exposed to duponol and then placed in five per cent trichloroacetic acid the shrinkage occurs until a rounded green mass containing colorless areas is formed. Small green rounded bodies appear in the rest of the cell. When the cells are transferred to duponol these structures disappear and the cell again shows a uniform green color.

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[1] This study was aided by grants from the National Institutes of Health, U. S. Public Health Service and the American Cancer Society.

A study of the growth of oysters under different ecological conditions in Great Pond. MADELENE E. PIERCE AND J. T. CONOVER.

A study of the seasonal growth of oysters under different ecological conditions was begun in Great Pond, Falmouth, Massachusetts, in the summer of 1953.

Great Pond, a tidal estuary 2.5 miles long and 0.5 miles wide, is composed of one large main basin which divides at its upper end into two narrow arms. One arm receives the Coonamessett River; the other arm, known as Perch Pond, receives only a small creek. At the outlet the main basin opens into Nantucket Sound; hence the salinity gradient ranges from fresh to 31 parts per thousand through a longitudinal section of the estuary.

On July 1, cages of two-year old oysters were placed at six stations chosen to include widely varying environmental conditions. The investigations were planned to cover at least one complete year, but during the winter most of the cages were stolen or their labels destroyed. Therefore the growing period on which measurements could be recorded was limited to an interval of 10 weeks, July 1–Sept. 9. Measurements of the length, width, depth, and volume of 240 oysters were made at the beginning and at the end of this period.

During the year monthly measurements of important physical and chemical properties included temperature, salinity, dissolved oxygen, and pH. During the limited survey period, at the six stations chosen, bottom temperatures varied from 22°–25° C. At one of two stations representing extremes in seasonal salinity ranges, located one mile from the Sound, bottom salinities varied between 29.7–30.5 parts per thousand, or 0.8; whereas at the other station, in Perch Pond, the range was 12.2–26.3 parts per thousand, or 14.1. The seasonal oxygen utilization and CO_2 production were particularly high at the brackish station in Perch Pond where the maximum increase in growth of the experimental oysters was recorded. The salinity gradient appeared to be the only suggestive limiting factor at some stations. Tidal water exchange was very limited at the station of maximum growth.

Oysters placed on muddy substrate died or showed little growth. Of those placed on sand, oysters living in the lower and more widely varying salinities showed the most growth.

With cages attached to heavier anchors and less conspicuous floats, these oyster experiments are being continued in the summer of 1954 along with productivity studies being made by members of the Woods Hole Oceanographic Institute.

Rhythms of O_2-consumption in the earthworm, Lumbricus terrestris. C. L. RALPH.

Oxygen-consumption of the earthworm, *Lumbricus terrestris*, was measured continuously for three consecutive semilunar periods, April 27 to June 9. The twenty-nine day period from May 12 to June 9, which was more fully analyzed, showed a bimodal diurnal variation in the rate of O_2-consumption, with maxima at 5 A.M. and 7 P.M. and with minima at 1 P.M. and 11 P.M. An analysis to demonstrate any primary lunar cycle for the same period showed one to be present which exhibited a major maximum at 11 P.M. and a minor maximum at 12 noon, with minima at 9 A.M. and 3 P.M. The last two semilunar periods were analyzed for diurnal variation and it was found that each showed essentially a bimodal pattern; the highest peak of the May 12 to 26 period occurred in the A.M. and the highest peak of the May 26 to June 9 period in the P.M. Analysis of the three semilunar periods separately for a primary lunar component showed in each case the presence of a rhythm possessing 12.4 hour cycles. In the first semilunar period maxima were nearly coincidental with lunar zenith and nadir; in the second period the maxima preceded these lunar times by 3 or 4 hours; and in the third period, the form of the cycles continued to become altered and the minima now were at the approximate times of lunar zenith and nadir.

Chromosome behavior during conjugation of mating types I and II of variety 1 of Tetrahymena. CHARLES RAY, JR.[1]

Chromosomal behavior of Elliott's mating types I and II of variety 1 of *Tetrahymena pyriformis* was studied by means of aceto-orcein-gelatin squash and by De Lamater's basic fuchsin staining schedule. The mating types have a haploid chromosome number of five.

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[1] Aided by grants from The University Center in Georgia and from The McCandless Fund of Emory University.

Meiosis takes place during the first two pre-zygotic divisions of the micronucleus. Pairing of the homologous chromosomes is accomplished just prior to or during the elongation of the "crescent" in prophase of the first division. Five bivalent chromosomes at diplotene are present as the crescent shortens from its maximum length. One or more chiasmata are present per bivalent. The five pairs of chromosomes condense and become oriented on the spindle. Five chromosomes are seen going to each pole at first anaphase. The second meiotic division follows quickly. Five chromosomes are seen at metaphase of second division. After the completion of the second division only one of the four micronuclear products divides again; the other three nuclei gradually degenerate. The functional nucleus is spindle shaped during prophase and located anteriorly, at the attachment membrane. The haploid number of five chromosomes is seen at third division. Anaphase is oriented antero-posteriorly in the conjugants and stretches nearly the length of the animal. Ten chromosomes are seen during the first postzygotic division of the synkaryon.

Histochemical studies of mesenteric structures. EVELYN KIVY ROSENBERG AND JOSEPH CASCARANO.

Neotetrazolium (NT), a water-soluble salt which becomes a purple, insoluble formazan upon reduction, has been used extensively within recent years as a histochemical tool for the demonstration of dehydrogenase activity in the living cell. Much work has been reported on the reaction of parenchymatous tissues such as kidney, adrenal, liver with this tetrazolium salt but little, if any, on small blood vessels, lymphatics, and peripheral nerves.

By the use of an osmotic, balanced medium we have been able to demonstrate endogenously (in living, viable tissues), a reaction between neotetrazolium and such blood vessels, lymphatics, and nerves in the mouse and rat. The medium finally decided upon is a 0.5% NT solution containing a modified Krebs formula made up of NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, KH_2PO_4 and buffered to a pH of 7.4 with phosphate. The tissues (*i.e.*, mesenteries or slices of organs) are incubated either aerobically or anaerobically for about three hours at 37° C. The metabolic reduction of NT is halted by immersion in 10% neutral formalin.

We have been able to distinguish readily arterioles, capillaries and venules, lymphatics (20–100 μ), and extremely delicate nerve fibers in mesenteries, as well as in organs; *i.e.*, bladder, tongue, uterus and fallopian tubes. The appearance of formazan in the blood vessels is one of discrete, granular deposition in the cytoplasm not only of the endothelium of blood vessels but also the muscular elements. The laying down of reduced tetrazolium in walls of lymphatics and nerve endings is also particulate. These structures may be readily distinguished from connective tissue fibers since the latter do not reduce tetrazolium.

With this tool, we are at present exploring methods for distinguishing changes in specific enzyme systems in different tissues following x-radiation.

The effect of various levels of x-irradiation on the gametes and early embryos of Fundulus heteroclitus. ROBERTS RUGH.[1]

The gametes of *Fundulus heteroclitus* are quite radioresistant as determined by the effect on subsequent development of the embryo; 25,000 r x-rays to the unfertilized eggs caused a wide range of abnormalities from quite normal to absence of forebrain. Eggs exposed to 100,000 r allowed some embryos to develop quite normally. These were probably haploids, developing parthenogenetically. 200,000 r to the eggs prevented their fertilization. Such an exposure to the eggs did prevent their fertilization. Such an exposure to the sperm did not prevent their motility nor their ability to fertilize normal eggs. While none of the resulting embryos from 200,000 r sperm ever hatched, many developed pulsating hearts connected with a circulatory system completely devoid of corpuscles. The sperm appeared better able to effect normal development following high levels of x-irradiation than did the eggs.

When the one- and two-cell stages were x-rayed to 300 r there was a stunting of development but no gross abnormalities, while 1,000 r caused complete suppression of the anterior neural differentiation. From 2 to 8 cells the maximum tolerable dose which allowed normal development was 500 r but at stage 11 (expanding blastula) even 1,000 r had no appreciable effect on development and it required 3,000 r to produce anterior end teratologies. Thus, the early fertilization and cleavage stages were the most radiosensitive.

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[1] The laboratory facilities for this study were provided by the Office of Naval Research.

In embryos which survived radiation exposure for some time the primary breakdown seemed to be in the circulatory system, followed by stasis in development affecting the central nervous and other systems. Pulsating hearts developed and were functional for some days, even without circulating cells. Cyclopia, complete absence of eyes and forebrain occurred while the more posterior structures appeared to be quite normal. Apparently the normal morphological movements at the time of gastrulation were affected and this caused structural abnormalities and final death of the embryos.

Antibiotics in relation to the survival of Spisula ova. VICTOR SCHECHTER.

The unfertilized egg cells of the clam, *Spisula solidissima*, were stored in sea water containing antibiotics in concentration ranging from 0.5 to 100 units per cc. While controls survived for a maximum of 32 hours, the ova in antibiotic-treated sea water endured for over 90 hours, and were then responsive to sperm. Dihydrostreptomycin and Chloromycetin were most effective. Aureomycin, Penicillin, Bacitracin, Polymyxin and Terramycin followed, in the order given. In some cases the maximum effect was obtained in the maximum concentration used, e.g., Chloromycetin; while with some of the other antibiotics the maximum effect was obtained at lower concentrations. With Aureomycin, for example, the maximum effect was obtained in a concentration of 25 units per cc. Cleavage could occur but was generally retarded in time, and to an extent inhibited. It was evident that the action of antibiotics consisted of more than a single effect, in that it involved the medium in which the eggs were contained, and the eggs themselves. These two effects were antagonistic in their action.

The effect of antibiotics upon Spisula sperm. VICTER SCHECHTER.

Spisula sperm placed in sea water containing antibiotics in concentration of 100 units per cc. generally lost their motility. In the case of Aureomycin and Polymyxin the effect was reversible. It was irreversible with Magnamycin. In Chloromycetin no impairment of motility was detected. Unlike the effect upon survival of Spisula ova, the action of antibiotics upon sperm may be taken to be directly upon the cells themselves, in its entirety, and unrelated to other biotic factors in the medium.

Evidence for active transport of water in Fundulus embryos. D. R. SHANKLIN.

The adult *Fundulus* exists in sea waters whose osmotic strength varies from two to four times that of the fish itself, the euryhaline state. Copeland (1948) has demonstrated the existence of an extra-renal osmoregulatory mechanism whose net effect is maintenance of such gradients in the face of frequently rapid changes. Developing *Fundulus* embryos are also subject to this euryhaline state. In the laboratory they exist and develop in the extremes of distilled water and at salt concentrations twice that of sea water. Evidence for water movement is best obtained by studies with deuterium oxide. The effect of osmotic change on salt balance, however, affords evidence for water movements. Comparison of salt balance in media whose colligative property coefficients are constant with those whose salt concentrations are constant, but heterosmotic, should reflect the order of osmotic strengths. This in fact does not occur. Since all such media are hypotonic to balance, dilution should occur, but in some instances concentration occurs. In those in which dilution does occur, the order is not related to the osmotic scale. These phenomena are more closely related to changes in the water gradient, as determined by densiometric studies—which property does not quantitatively parallel the tonicity. Comparisons of heterosmotic and anisohydric states with normals in presence of glycolytic inhibitor fluoride shows a connection between energy mechanisms and water movement. In presence of fluoride there is egression of water with the osmotic gradient, and ingression against the gradient in the absence of fluoride. These responses permit the embryo to maintain an osmotic pressure 20–25% of the surrounding medium.

The action of trypsin on Arbacia and Echinarachnius eggs in homologous and cross fertilization. ALBERT TYLER AND CHARLES B. METZ.

Trypsin treatment dissolves the gelatinous coat (fertilizin) of unfertilized sea urchin eggs (Tyler and Fox, 1940); it inhibits formation of the fertilization membrane (Kunitz, 1932) and renders the eggs more readily cross fertilizable (Hultin, 1948; Bohus Jensen, 1953). These effects initiated the present study.

Arbacia eggs were treated with 0.05% crystalline trypsin for periods of ½ to 3 hours and aliquots inseminated with serial dilutions of *Echinarachnius* sperm. The concentration of sperm required to yield equal percentages of fertilization in treated and control eggs was then compared. This revealed that trypsin-treated eggs required less than 1/27 the amount of sperm necessary to obtain comparable fertilization of control eggs. Similarly in the reciprocal test (*Arbacia* sperm × *Echinarachnius* eggs) treated eggs required less than 1/9 as much sperm as controls. The experiments also showed that fertilizability of trypsin-treated eggs by *homologous* sperm was markedly reduced. Treated eggs (*Arbacia*) required 50 × the amount of sperm necessary for the controls. Fertilization and cleavage also occurred in the trypsin solution. *Arbacia* eggs with or without trypsin treatment failed to cross fertilize with *Asterias* sperm. This result suggests that trypsin does not extend cross fertilizability to new combinations but rather facilitates crosses that can occur independently.

Since trypsin obviously removes most of the fertilizin (gelatinous coat) from the eggs, it seemed desirable to determine whether or not some remains as part of the surface. It was found that the addition of antifertilizin (which agglutinates control eggs) inhibits fertilization of both trypsin-treated and control eggs and may agglutinate trypsin-treated eggs. This suggests that at least some fertilizin remains on the surface of trypsin-treated eggs.

Unlike *Strongylocentrotus* and many other species, *Arbacia* sperm following treatment with fertilizin does not suffer a marked loss in capacity to fertilize normal eggs. Since trypsin renders *Arbacia* eggs less readily fertilizable, such eggs appeared to provide a more sensitive test for an effect of *Arbacia* fertilizin on the fertilizing power of homologous sperm. This proved to be correct, for the concentration of fertilizin-treated sperm required to fertilize trypsin-treated eggs was 100 × that of the untreated sperm. This result is interpreted as a decrease in available receptor groups on both the egg (fertilizin) and sperm (antifertilizin).

Loss of radioactivity from tissue sections during histological processing. W. S. VINCENT.

Intracellular distribution of certain substances which will incorporate radioisotopes is possible through the use of radioautograph techniques. Semi-quantitative estimates of the specific activity of certain compounds have been made through the use of enzymatic digestion of tissue sections which have been so labelled, and the counting of reduced silver grains in the emulsion before and after digestion. The extracting effects of aqueous solutions on P^{32}-containing compounds in sections of Amblystoma larvae exposed to 100 μc of P^{32} are given below. The data are expressed as per cent of counts in the section before removal of the paraffin which remain after the indicated treatment. Removal of paraffin: 92; hydration (through alcohols to water): 80; RNAase (two hours, 37°): 25; RNAase control (H_2O): 56; *N* HCl (14 minutes, 60°): 35; HCl controls (H_2O): 66. A similar pattern of loss of radioactivity from starfish oocytes has also been demonstrated.

The nature of the radiophosphorus-containing materials which are soluble in the aqueous solutions has not yet been demonstrated. These data suggest that considerable caution is required in quantitative interpretation of radioautographs.

Induction of a persistent modification in the daily rhythm of O_2-consumption in fiddler crabs.[1] H. M. WEBB, M. F. BENNETT AND F. A. BROWN, JR.

Fiddler crabs of two species, *Uca pugnax* and *Uca pugilator,* were subjected to three cycles of illumination by night and darkness by day. At the end of this time, and again at the conclusion of the experiment, it was ascertained that the daily rhythm of color change was inverted. The form of the daily rhythm of O_2-consumption of these "reversed" animals under constant conditions was compared with that of controls for two consecutive fifteen-day periods. It was found that the ratio of the average rate of O_2-utilization for the hours between 5 and 10 A.M. to the average rate for the 5 to 10 P.M. hours was about 35% higher in the light-reversed animals than in the controls. This is consistent with the view generally held that the hormone normally involved with the darkening of the body is a depressant for O_2-consumption and that O_2-uptake is lowered at the time of its liberation into the blood and elevated when the titre of this hormone is diminished. Though this described modification occurred, the general form of the daily rhythm, with its tendency for maxima to be present at 3 A.M., 9 A.M., and 3 P.M., with the principal minimum during the evening hours, appeared otherwise unaltered by the light treatment.

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[1] This work was supported in part by contracts NONR-09703 and NONR 122803 with the Office of Naval Research.

Variations in x-radiation sensitivity of different stages of growth in Paramecium.[1] RALPH WICHTERMAN.

After specimens from a given clone of *Paramecium caudatum* are irradiated, considerable variation in x-ray susceptibility is seen to occur depending primarily upon the phases and stages of growth of the organisms when environmental and technical factors are maintained fairly constant during and after irradiation.

Nylon syringes of two-cc. capacity were used as irradiation chambers. In a typical experiment, 200 specimens representing one phase of growth were placed in each syringe. Four syringes containing the different stages were irradiated simultaneously with 250 kiloroentgen. After irradiation, specimens were expressed into sterile spot-plates and kept in moist chambers for observation.

The LD 50 of *Paramecium caudatum*, 24 hours, is approximately 340 kr when fully differentiated, well-fed, vigorous specimens are irradiated from a culture which has reached the peak of maximal division rate. Such specimens when irradiated with 250 kr prove to be more radioresistant than vegetative ones from older cultures containing more transparent, thinner, less vigorous animals in which feeding activity and fission rate are greatly reduced. Twenty-four hours after irradiation, 66 per cent of the vigorous, well-fed specimens survived in contrast to 38 per cent of the other type. Forty-eight hours after irradiation, more specimens in each group succumbed resulting in 49 per cent survival in the former and 21 per cent in the latter. Recovery of x-radiation effects is faster in the survivors of the well-fed, vigorous group than in the other even when both groups of survivors are treated in the same manner.

Based upon these and other experiments, attention is called to the fact that some immobilized paramecia may, for hours, appear dead during the first day after irradiation only to recover after the first 24-hour period. More reliable results may be obtained for *Paramecium* and possibly other Protozoa by determining the LD 50 values for a 48-hour period following x-radiation instead of the commonly used 24-hour period. Also, attention is called to the significance of physiologic conditions and stages of growth of the Protozoa to be irradiated.

An antimitotic substance from muscle.[2] WALTER L. WILSON AND L. V. HEILBRUNN.

According to Bullough, muscular exercise tends to inhibit mitosis in the skin of mice. This may be due to the production of an antimitotic substance by contracting muscle.

As a result of the work of Most, frog muscle is known to produce a heparin-like substance as a result of repeated contractions, and accordingly we began our experiments with muscles of the frog. These experiments are complicated by the fact that in order to use marine eggs for test objects, the salt solution bathing the frog muscles has to be concentrated so that the final tonicity is equal to that of sea water. Accordingly we turned to lobster muscle, using the eggs of the worm Chaetopterus to test for antimitotic activity. The tail muscles of lobsters were stimulated electrically. After the muscles, following repeated stimulation, were no longer able to contract, they were cut out of the animal, placed into sea water, and teased apart. After a half hour, the sea water which had been in contact with the muscles was found to be rich in a substance which prevented division of the eggs. Control, unstimulated muscles also give off this substance but in much lower concentration. The antimitotic substance from the fatigued muscles keeps the protoplasm in a fluid state—that is to say, it prevents the mitotic gelation. It is a heat-stable substance and resists boiling. Further studies on its chemical properties are planned.

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[1] Aided by a grant from the Committee on Research, Temple University and performed under Contract NR 135–263 between the Office of Naval Research, Department of the Navy and Temple University.

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Inhibition of intermedin-induced darkening of frog skin (Rana pipiens) by tetrazolium salt. Paul A. Wright.

An interesting physiological property of triphenyltetrazolium chloride (TTC) is its ability to inhibit darkening (dispersion of melanophoral pigment) of isolated frog skin as induced by intermedin. A solution of 0.1% (0.003 *M*) TTC in Ringer's fluid totally prevents the darkening response to an optimal dosage of intermedin (20 units); 0.001 *M* solution is at first inhibitory (30 minutes) and then permits partial darkening; 0.0001 *M* TTC is ineffective. The salt appears to affect the responsiveness of the melanophores and does not merely inactivate the hormone, since a 0.003 *M* solution is just as effective in inhibiting 40 or 400 units of intermedin as it is 2 or 20. The inhibition is immediate and the formation of formazan appears to have no part in the reaction; in fact, photoreduced TTC and freshly prepared solutions are equally potent in preventing darkening. Succinate (0.001 *M*, pH 7.4) did not protect the skin against the action of tetrazolium. There is some indication that glucose (0.01 *M*) can partially release the inhibition imposed by TTC, but levulose and galactose had none of this ability.

TTC did not alter the blanching pattern, which follows darkening, in either Ringer's fluid or solutions of epinephrine, but skins which had been maximally darkened with intermedin returned promptly to the blanched state when TTC was added, even in the presence of an optimal dosage of intermedin (20 units). Addition of TTC at any point during an intermedin-induced darkening causes the melanophores to cease pigment dispersion and begin at once the process of melanin concentration. Further work is in progress to elucidate the mode of action of tetrazolium salts.

Native protein structure and a tetrahedral motif.[1] Dorothy Wrinch.

Crystal data for many globular proteins direct attention to some striking properties including a spread over all 11 possible classes, cases of pseudo-symmetry, and twins and intergrowths related to the crystal class. The structures of the silicates etc. suggest that these properties may be a consequence of a motif with a tetrahedral quality in the skeletons of protein molecules. This we find in the five atom motif, $N—(H—)C_{\alpha}(—C_{\beta})C$, which may be playing for protein molecules a part formalistically similar to that played by the SiO_2 motif in cristobalite, the common feature being the setting of motifs to their fellows with reference to a single set of cubic axes. Picturing such motifs assembled with symmetry 231, R-groups could be inserted with symmetry 231 or 22 or 3 or 2 or 1. Assuming mutual affinities of such structures when apposed in parallel, with or without one-quarter turns about cube edges and one-sixth turns about cube diagonals, explanations of the properties could be given.

LALOR FELLOWSHIP REPORTS

I. A search for L-spinacine in dogfish liver. II. An investigation of the constituents and function of the hypobranchial gland of certain Muricidae. Bruce N. Ames.

L-spinacine (imidazo-tetrahydropyridine carboxylic acid) was reported to be present in large amounts in shark (*Acanthias vulgaris*) by Ackermann. This compound is of interest

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[1] This work is supported by the ONR under contract with Smith College.

because of its close structural relationship to both histidine and purines. We have synthesized spinacine from histidine by the procedure of Neuberger, and have been able to localize it on paper chromatograms by spraying the paper with diazosulfanilic acid, a reagent for imidazoles. No spinacine could be detected on chromatographing extracts of dogfish (*Squalus acanthias*) liver.

Urocanylcholine (murexine) was found in the hypobranchial gland of *Murex trunculus* and several related Mediterranean snails by Erspamer. It is closely related in structure to acetylcholine and has a pronounced curare-like action. The function of the hypobranchial gland in snails is not understood. We have chromatographed the glands of several local Muricidae in an attempt to correlate the feeding habits of the snails with the presence of urocanylcholine. Extracts of the glands were chromatographed on paper and the urocanylcholine spot eluted and read in the Beckman. In addition to a strong ultra-violet absorption, urocanylcholine may be indicated on chromatograms by a diazosulfanilic acid spray. Thais and Urosalpinx contain large amounts of urocanylcholine in the hypobranchial gland but not in other organs. Thais glands also contain several fluorescent compounds and diazo-reacting spots distinct from murexine, histidine, and urocanic acid. Each species of snail gives a characteristic pattern when a particular organ is chromatographed on paper and the chromatogram is viewed with ultraviolet light. No evidence as to the function of the hypobranchial gland has been obtained.

A study of some proteolytic enzymes during the development of Ilyanassa obsoleta. Jack R. Collier.

The hydrolytic activity of three peptidases and three proteinases was measured in the egg and at subsequent stages of development of Ilyanassa. The relative activity of all enzymes studied is expressed as microliters of 0.05 *N* HCl necessary to titrate the free carboxyl groups which were released by the enzymatic hydrolysis.

The relative activity of glycylglycine dipeptidase and aminotripeptidase of the Ilyanassa egg was equivalent to 0.11 and 0.22 microliters of 0.05 *N* HCl per egg, respectively. By the fifth day of development (at 19 degrees C.), at which time a rapid growth of the organ primordia begins, the activity of each of these peptidases had increased by about 55 per cent; by the tenth day, when the veliger larva is fully developed, the glycylglycine dipeptidase activity was about 700 per cent greater than the activity found in the egg. Alanyl-l-histidine dipeptidase activity could not be detected in the egg or at any subsequent time during development.

The hydrolytic activity of cathepsin II was not detected at any time prior to the fifth day of development. The activity of cathepsin II in the five-day-old embryo was equivalent to 0.05 microliters of 0.05 *N* HCl per embryo and by the sixth day of development a two-fold increase had occurred. This increase in catheptic activity continued during the remaining four days of development and by the tenth day the cathepsin activity was equivalent to 0.36 microliters of 0.05 *N* HCl. The hydrolytic activity of pepsin was not detected in the egg or in the four-day-old embryo; however, the pepsin activity of the veliger larva was equivalent to 0.56 microliters of 0.05 *N* HCl per larva. The trypsin activity of the veliger was equivalent to 0.13 microliters of 0.05 *N* HCl per larva.

The intracellular localization of the alanylglycine dipeptidase activity in the egg of Ilyanassa obsoleta. Jack R. Collier.

The Ilyanassa egg was fragmented by centrifuging in gum arabic for 10 minutes at a centrifugal force of about 2,000 times gravity. The centrifugal fragment contained all of the large yolk platelets and a small amount of hyaline protoplasm, whereas the centripetal fragment consisted of an oil cap and most of the hyaline protoplasm. The centripetal half represented 50.4 per cent of the total volume of the egg and the centrifugal fragment 49.6 per cent. The percentage distribution of the alanylglycine dipeptidase activity was 69.6 per cent in the centripetal fragment and 21.2 per cent in the centrifugal fragment.

The volume of the yolk platelets in the Ilyanassa egg was measured by homogenizing the egg and packing the platelets in a capillary tube with subsequent measurement of the volume of the capillary occupied by the yolk platelets. From these measurements it was found that the yolk occupied 33.4 per cent of the total volume of the egg. The volume of the oil cap was determined by calculating the volume of the spherical segment which it occupied in the centrifuged egg and was found to be 10.5 per cent of the total volume. Thus, the hyaline protoplasm (plus its finer inclusions) was found to occupy 56.1 per cent of the total volume of the egg.

Considering that the centrifugal fragment contained all of the yolk platelets plus some hyaline protoplasm, and that it represented 49.6 per cent of the total volume of the egg the percentage of the hyaline protoplasm in the centrifugal fragment was found to be 16.2 by subtracting the volume of the yolk contained in the egg from the volume of the centrifugal fragment. The percentage of hyaline protoplasm in the centrifugal fragment is in close agreement with the percentage of the dipeptidase activity in the centrifugal fragment. Thus, it seems probable that the alanylglycine dipeptidase activity is contained in the hyaline protoplasm and/or its finer inclusions.

Attempt at isolation of desoxyribonucleoprotein from pneumococci. H. EPHRUSSI-TAYLOR.

Although several types of desoxyribonucleoproteins have been isolated from higher organisms, no evidence has ever been obtained demonstrating the existence in bacteria of desoxyribonucleic acid in a protein-bound form. In collaboration with Dr. Jay Barton, an attempt has been made to isolate from pneumococcus a desoxyribonucleoprotein, employing the technique described by Mazia and Bernstein. Employing distilled water extraction of frozen and thawed bacteria of a streptomycin-resistant strain of pneumococcus, it has been possible to isolate a material which is endowed with transforming activity (conversion of streptomycin-sensitive pneumococci into resistant ones). Characterization of the extracted material is as yet incomplete. Analyses show the extracts to contain appreciable amounts of protein, ribonucleic acid and desoxyribonucleic acid, but the extent to which the components are bound to each other remains to be determined. No material having the solubility of nucleohistone was found. Quantitative studies of transforming activity of extracts strongly suggest that the transforming factor is present in bound or aggregated form, since activity increases 100–1000 fold upon dilution. No inhibitory substances could be detected, which otherwise might have accounted for the low activity of undiluted extracts.

Fine structure in the oocyte nuclear membrane. JOSEPH G. GALL.

Nuclear membranes from isolated oocyte nuclei of the starfish *Henricia* were spread flat on plastic "Formvar" films, fixed in 1% osmium tetroxide, dried, and observed with the electron microscope. The membrane consists of a thin continuous sheet with raised annuli scattered on its cytoplasmic side. The annuli are roughly 1000 Å in diameter with a center about 400 Å across. In thin sections of osmium-fixed oocytes the continuous sheet appears as a fine line about 150 Å thick. The annuli are not easily distinguished from the cytoplasm in transverse sections of the nuclear membrane, but are apparent in areas cut tangentially. Annuli of similar dimensions have been seen in sectioned oocytes of the annelid *Chaetopterus,* and in spread nuclear membranes from oocytes of the frog *Rana,* the newt *Triturus,* and an unidentified minnow. These observations suggest that the nuclear membranes of widely divergent forms may be constructed on a common plan.

The effects of various agents have been tested on unfixed nuclear membranes of *Triturus.* Some solutions appeared to digest the membrane (pepsin, trypsin), while others had little effect on its fine structure (distilled water, various concentrations of sodium chloride up to 1.0 *M,* phosphate buffer at pH 7.0, equal parts of ether and alcohol, and solutions containing calcium or from which this ion had been removed by the chelating agent "Versene"). The nuclear membrane is therefore composed, at least in part, of relatively insoluble protein.

A further study of isometric mechanical responses of the anterior byssus retractor muscle of Mytilus edulis to electrical stimuli and mechanical stretch. WILLIAM H. JOHNSON.

Winton has shown that following direct current stimuli, the anterior byssus retractor muscle of *Mytilus edulis* relaxes very slowly and shows an increase of tensile "viscosity" which can be reversed by a burst of alternating current or short D.C. pulses. The long-lasting D.C. response is cathodal, as shown by Fletcher. Thus, in the present work, a Taylor triangular electrode was used in an attempt to produce as large a cathode as possible at the muscle surface. It was found that a) if the electrode is made cathodal, the response to single pulses was large, if anodal, the response is small; b) a typical D.C. response is obtained at pulse durations as short as 20 msec.; c) the response becomes greater as the pulse duration is increased; d) these responses will sum, until, at high repetition rates, a Winton "tetanic" response is obtained. Quick stretches applied to the muscle showed the muscle to be more plastic following "tetanic" stimuli than following D.C. stimuli, as found by Winton. Even at muscle lengths below rest length, the stiffness increases following D.C. stimuli. This increase is similar to, but smaller than, the increase in stiffness seen during iodoacetate rigor. The "unlocking" or "plasticizing" action of the tetanus takes place during a short interval following the cessation of the stimulus. The hypothesis is advanced that these visco-elastic changes may be produced by variations in intrafibrillar concentrations of ATP, similar to the effects found by Weber using glycerinated psoas fibers. In this muscle, the stiffness can be increased with little concomitant contraction by adjusting the repetition rate of short D.C. stimulating pulses; thus the visco-elastic change might be a major physiological response in such holding muscles.

Some observations on bioluminescences. BERNARD L. STREHLER (with kind assistance of R. Cahn and M. Owens).

Several bioluminescent systems have been examined with respect to general and more specific properties. The chemiluminescence of green plants has been studied with respect to the decay curves of this luminescence during the early period after the illuminating flash. In addition, about 30 species of marine and land plants including brown, red and green algae, conifers, and a variety of monocots and dicots, have been examined for the occurrence and intensity of this luminescence. All plants examined exhibited this luminescence.

The major effort was centered on Mnemiopsis bioluminescence. Many prior observations have been confirmed. Among the newer observations are the following: Stable luminous powders can be prepared by low temperature lyophylization; chemiluminescence can be elicited from cell suspensions by a great variety of physical and chemical agents including mechanical stimuli, electrical shock, detergents, alcohol, freezing, etc. No luciferin-luciferase reaction has thus far been demonstrated. Factors necessary for bacterial or firefly luminescence were ineffective in this system. With the supply of stable powders obtained this summer it is hoped that the biochemical investigations can be carried further during the coming winter.

Large amounts of isolated luminous material from ctenophores have been extracted with organic solvents in an attempt to obtain the pigments responsible for bioluminescence and for the photoinactivation of the luminescence of the intact ctenophore or cell suspensions obtained from it.

Finally, with the hypothesis in mind that light might trigger an internal chemiluminescence which would act as an amplifier in visual receptors, squid and Fundulus eyes were examined with a phosphoroscope-quantum counter combination. No delayed luminescence was observed, indicating that the hypothesis is probably incorrect.

P^{32} incorporation into starfish oocyte nucleoli. W. S. VINCENT.

The incorporation of P^{32} into various fractions of the starfish oocyte nucleolus has been studied by direct analysis of isolated nucleoli and by autoradiography. P^{32} enters the nucleolus at a linear rate over a nine hour period. The relative specific activities of nucleolar fractions are as follows: whole nucleolus, 1.0; acid-soluble P, 3.0; RNAP, 0.5; protein P, 0.2. The relative specific activity of the whole egg P and cytoplasmic RNAP were 1.0 and 0.5, respectively. The dilute acid-soluble fraction contains non-dialysable nucleotides of relatively high specific activity. Semi-quantitative grain counts of autoradiograms of nucleoli in tissue sections indicate that the RNA of *in situ* nucleoli has a specific activity of 100 to 1000 times that of the cytoplasmic RNA. Ultraviolet absorption measurements on isolated and *in situ* nucleoli have shown that about 10% of the ribonuclease-removable absorption is lost during isolation. Preliminary studies of autoradiographs of aliquots of egg homogenates taken during various intervals of the isolation procedure suggest that considerable radioactivity is lost from the nucleoli during the early steps of isolation.

These data suggest that the starfish nucleolus contains two types of RNA. One type is loosely bound and metabolically labile, as is indicated by the concentration of radioactivity in the material lost during isolation of the nucleolus. The other type incorporates P^{32} quite slowly, and is tightly bound to the nucleolar structure.

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THE SENSITIVITY OF DEVELOPING HABROBRACON TO OXYGEN[1]

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Injury from high tensions of oxygen has been reported for a wide range of plants and animals. These have included chromosome breakage (Conger and Fairchild, 1952) and numerous non-nuclear changes (Stadie, Riggs and Haugaard, 1944). Among insects, toxicity from oxygen, as manifested by a decrease in wing beat frequency and reduced viability, has been shown for adults of *Drosophila azteca* exposed to up to ten atmospheres of oxygen (Williams and Beecher, 1944). *Drosophila melanogaster* exposed as embryos exhibited a slight lag in development, but without concomitant effects on viability (Glass and Plaine, 1952). During investigations on the parasitic wasp, *Habrobracon*, it was found that pupal development was arrested by high tensions of oxygen. Since reports of such effects are meager, it was decided to investigate this phenomenon in more detail.

MATERIALS AND METHODS

The methods of rearing *Habrobracon* and of experimentation have appeared in previous publications (Clark and Mitchell, 1951). Females from stock No. 33 were crossed to males from stock No. 1. Diploid daughters were obtained from the fertilized eggs and haploid sons from unfertilized eggs. The male and female offspring were grown together in the same culture and treated at the same stage of development. All cultures were raised at 30° C.

The stages of development at which *Habrobracon* were treated and the chronological ages that coincide with these stages are shown in Figure 1. "Larva-in-cocoon" (3–4½ days) is the period from the enclosure of the larva in the cocoon to the appearance of the meconium; "prepupa" (4½–5½ days) is the stage from the egestion of the meconium to the presence of external appendages.

The wasp cultures were exposed to air, oxygen, nitrogen, and carbon dioxide in chambers about 100 cm.^{3} in volume. The gases were obtained from commercial cylinders. No attempt was made to remove impurities since previous investigators such as Conger and Fairchild (1952) found no objectionable effects from trace contaminants in commercial cylinders.

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The deleterious effects were measured by determining the incidence of adults emerging from cocoons (the eclosion ratio), the occurrence of structural abnormalities among these adults and the arrest of development.

RESULTS

Groups of individuals that ranged from the larva-in-cocoon stage to the pigmented pupal stage were exposed to one atmosphere of oxygen or of nitrogen for one hour at 30° C. Groups exposed to oxygen as larvae in cocoons or as young prepupae showed no injurious effects. Groups treated as older prepupae or as pupae were injured, white pupae (6–6½ days) being the most sensitive (Table I, Fig. 1). The exposure to oxygen decreased the incidence of individuals developing to the adult stage and emerging from cocoons. Arrest of development usually occurred during the pigmented pupal stage, although such pupae remained alive for at least two weeks after eclosion of controls. An appreciable number of the adults which did emerge show wing and antennal abnormalities. The eclosion per cent for females was significantly less than for comparable males. None of the deleterious effects described for oxygen were found following exposure to nitrogen at any stage of development. The eclosion ratios for nitrogen-treated groups were not significantly different from air controls.

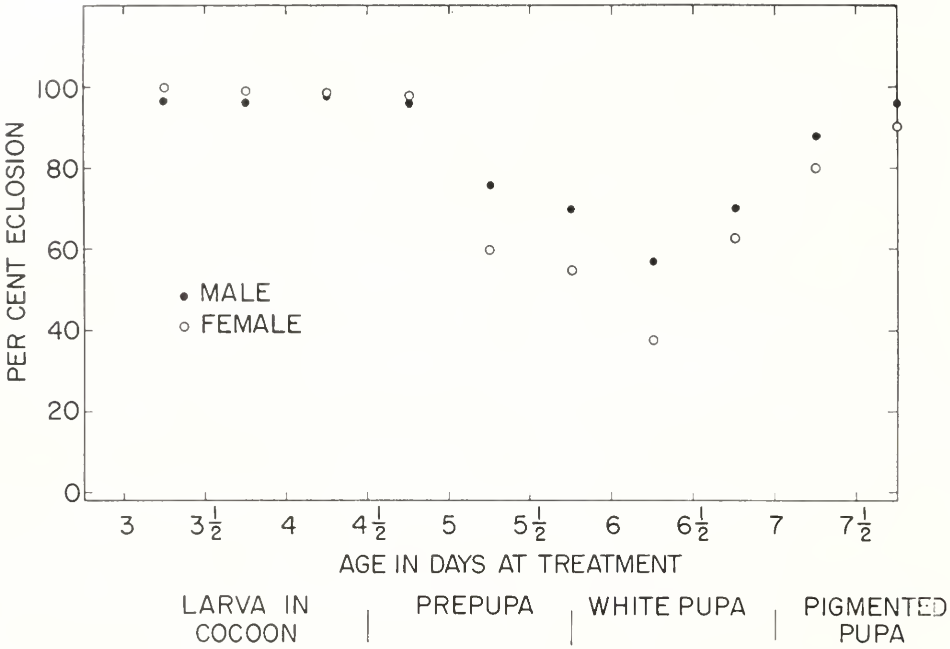

FIGURE 1. The relation of stage of development to oxygen sensitivity in Habrobracon. The deleterious effect is expressed as per cent eclosion following exposure to oxygen (100 per cent) for one hour. Data from Table I.

The preceding experiment shows that the oxygen sensitivity of *Habrobracon* varies markedly during development. Next, groups of individuals in a sensitive

TABLE I

Eclosion ratios for males and females after exposure to oxygen or nitrogen at different stages of development

Age (days) at treatment	Oxygen				Nitrogen			
	Males		Females		Males		Females	
	eclosion ratio	no. counted	eclosion ratio	no. counted	eclosion ratio	no. counted	eclosion ratio	no. counted
3–3½	.97	29	1.00	11				
3½–4	.96	182	.99	142	.90	50	.92	39
4–4½	.98	186	.98	173	.98	43	.96	49
4½–5	.96	185	.98	168	1.00	36	.92	57
5–5½	.76	286	.60	270	.96	50	1.00	33
5½–6	.70	354	.55	404	.98	58	.97	36
6–6½	.57	402	.38	452	.99	65	.98	41
6½–7	.70	371	.63	361	.93	44	.94	33
7–7½	.88	56	.80	39	.96	49	.97	35
7½–8	.96	50	.90	33	.92	53	.95	42
air controls	.96	439	.97	340				

period (white pupal stage) and during a resistant period (larva-in-cocoon stage) were exposed to air, oxygen, nitrogen or carbon dioxide at one or at two atmospheres pressure. The groups were exposed at one atmosphere for 15 minutes or at two atmospheres for 5 minutes.

Eclosion ratios following treatment of the larva-in-cocoon stage with air, oxygen, nitrogen or carbon dioxide at one or at two atmospheres indicate no deleterious effects on development or eclosion (Table II). Following exposure of white pupae to one atmosphere of nitrogen, eclosion is not significantly lower than air controls, but after one atmosphere of oxygen or of carbon dioxide eclosion is significantly lower (Table II) and after two atmospheres of oxygen or carbon dioxide there is a still greater decrease in the incidence of adults eclosing. A decrease in per cent eclosion occurs following treatment in two atmospheres of air over one atmosphere of air. Treatment in two atmospheres of nitrogen shows a non-significant decrease in eclosion for males and a small but significant decrease for females. Repetition of these experiments showed no decrease in per cent eclosion following treatment with nitrogen at two atmospheres versus one atmosphere, but did show marked differences between one and two atmospheres for air, oxygen and carbon dioxide. The difference shown for nitrogen between female pupae treated at one and at two atmospheres (Table II) is therefore spurious. Since treatment in two atmospheres of nitrogen shows no deleterious effects, it would seem that pressure itself is not responsible for the effects. The eclosion ratios following treatment in one atmosphere of oxygen or carbon dioxide are less for females than for males.

The development of white pupae exposed to two atmospheres of oxygen was arrested immediately. They remained as white pupae for one week, then started to develop pigmentation. These pupae remain alive in this arrested condition for at least three weeks after controls have emerged as adults. Observations of the arrested pupae were not continued after this period. Of comparable carbon

TABLE II

Eclosion ratios for males and females after exposure to air, oxygen, nitrogen or carbon dioxide

Gas	Sex	Larva-in-cocoon stage				White pupa stage			
		1 At.		2 At.		1 At.		2 At.	
		eclosion ratio	no. counted	eclosion ratio	no. counted	eclosion ratio	no. counted	eclosion ratio	no. counted
Air	♂	.91	54	.77	44	.90	93	.64	55
	♀	.92	64	.82	50	.90	112	.68	111
Oxygen	♂	.92	52	.85	54	.55	145	.03	77
	♀	.92	75	.86	56	.35	124	.01	74
Nitrogen	♂	.87	76	.91	45	.92	102	.82	60
	♀	.87	103	.90	40	.93	99	.75	87
Carbon dioxide	♂	.85	106	.83	40	.67	161	.23	64
	♀	.91	155	.95	59	.46	213	.09	57

dioxide-treated pupae, some continued to develop to the pigmented pupal stage before becoming arrested while others lagged for a few days as white pupae. Oxygen-treated pupae appear to be affected more than comparable pupae exposed to carbon dioxide.

Following exposure for five minutes to two atmospheres of nitrogen or to two atmospheres of oxygen, 25 white pupae were placed in each Warburg flask and oxygen uptake measured. Oxygen uptake was recorded in terms of the displacement of the Brodie's fluid, which serves sufficiently well for comparison of the effects of the gases. Upon repetitions of this experiment flasks used to measure oxygen-treated pupae were used to measure the oxygen consumption of nitrogen-treated pupae. Since the same results were obtained in all cases, errors due to slight volume differences in flasks or in the bore of the manometer are irrelevant. Neither two atmospheres of nitrogen nor one atmosphere of oxygen affected oxygen uptake. Two atmospheres of oxygen, however, resulted in an immediate and marked decrease in oxygen uptake (Fig. 2). Exposure to two atmospheres of carbon dioxide produced only a slight decrease in oxygen uptake. No decrease in oxygen uptake was observed following exposure of larvae-in-cocoons to oxygen or carbon dioxide at normal or increased pressure. The foregoing experiment shows a marked and immediate decrease in the oxygen uptake of white pupae exposed to two atmospheres of oxygen. Further work of a more quantitative nature has been planned.

DISCUSSION

Most studies have shown that the metabolic rate of immature insects is lowered by exposure to low tensions of oxygen (Wigglesworth, 1950). Effects of high oxygen tension on development have been reported by Glass and Plaine (1952) who found a slight lag in eclosion from cocoons without concomitant effects on viability for *Drosophila* exposed as embryos. The present work shows that a decrease in metabolism can be obtained by exposure to high tensions of oxygen. Pupae of *Habrobracon* are arrested in their development following such treatment and oxygen consumption is reduced. The development of pupae of the *Cecropia* silkworm is slightly delayed following exposure to 100 per cent oxygen for 21 days and slightly abnormal adults are produced (Schneiderman and Williams, 1954). In neither *Drosophila* nor *Cecropia* has treatment with oxygen at higher pressures been reported.

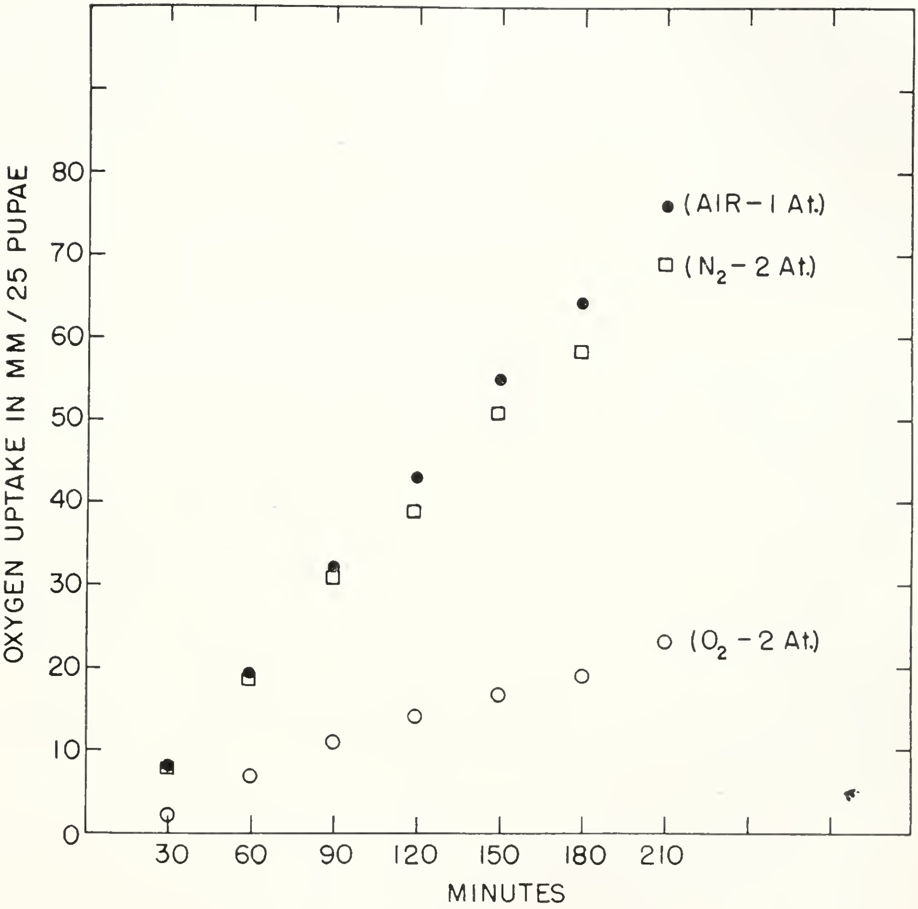

FIGURE 2. The oxygen consumption of white pupae following exposure for five minutes to two atmospheres of oxygen and of carbon dioxide. Ordinate: millimeters displacement of fluid in the arm of the manometer. Twenty-five pupae were used in each flask.

Work on animals as unrelated as rats, turtles and *Drosophila* adults has shown that younger animals are more resistant to injury by oxygen than older animals (Williams and Beecher, 1944). In *Habrobracon* this sensitivity is stage-dependent, since white pupae are more sensitive than the younger larvae or the older pigmented pupae. The sensitivity may be also sex-dependent since males are more resistant than females.

The oxygen-sensitive, white pupal, stage is also injured by exposure to carbon dioxide and the oxygen-resistant, larva-in-cocoon, stage is unaffected by carbon dioxide. The injury from carbon dioxide is probably not due to anoxia because the duration of exposure was short. An effect from carbon dioxide is indicated since injury from two atmospheres of carbon dioxide is greater than from one atmosphere. Williams and Beecher (1944) have shown for *Drosophila* that the toxic effects from oxygen are increased as the carbon dioxide tension is increased. The present observations can do no more than to point out the extreme sensitivity of *Habrobracon* white pupae to oxygen and to carbon dioxide. Further work is necessary to explain the mechanisms involved.

The authors thank Dr. John Buck of the National Institutes of Health and Dr. Robert King of the Brookhaven National Laboratory for their careful and vigorous criticism of this manuscript.

SUMMARY

1. Groups of *Habrobracon* were exposed during post-embryonic development to air, nitrogen, oxygen and carbon dioxide and studied with regard to development and oxygen uptake. Deleterious effects from oxygen and from carbon dioxide were observed after exposure at the white pupal stage, but not after exposure at the larva-in-cocoon stage. No deleterious effects from exposure to nitrogen were found.

2. Exposure of white pupae to one atmosphere of oxygen or carbon dioxide caused cessation of development at the pigmented pupal stage in a high proportion of the individuals. The incidence of emergence from cocoons as adults was higher for males than for females. Adults with wing and antennal abnormalities occurred. Exposure to two atmospheres of oxygen or carbon dioxide resulted in greater deleterious effects on development than did exposure to one atmosphere.

3. The oxygen uptake of unpigmented pupae exposed to two atmospheres of oxygen is markedly reduced. No such reduction was observed following treatment with two atmospheres of nitrogen or carbon dioxide or one atmosphere of oxygen.

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STUDIES ON THE ACROSOME.

III. EFFECT OF CALCIUM DEFICIENCY[1]

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The idea that calcium is necessary for fertilization was clearly formulated by Jacques Loeb (1914, 1915), who suggested, as a possible explanation, that it might be an ingredient of a sort of cement which holds the sperm to the egg surface in the first stage of sperm attachment.

A very different and more complicated role in the fertilization reaction has been attributed to the calcium ion by Heilbrunn, as a corollary of his general theory of stimulation (for summary, see Heilbrunn, 1937, chaps. IX and XXXVII). In particular the work of his student, Moser (1939), showed that the breakdown of the cortical granules of sea urchin eggs, which is the precursor of membrane elevation and subsequent developmental processes in normal fertilization and artificial parthenogenesis, does not occur when the eggs are activated in oxalated or citrated media. Yamamoto (1954), pushing this analysis a step further, has found that in teleost eggs calcium ions are necessary for the propagation of an invisible "fertilization-wave" which precedes and sets off the cortical changes. Sugiyama (1953) has shown that a similar wave of excitation occurs on fertilization in the sea urchin egg.

This demonstrated importance of calcium for the first detectable change in the egg cortex at the time of activation, together with the fact that spermatozoa are normally active and attack the unfertilized egg surface vigorously even in the absence of calcium, has led to a general assumption that the failure of fertilization in Ca-free media is due to the inability of the egg to respond to the stimulus of the spermatozoan, rather than to any reduction of spermatozoan stimulating capacity in the absence of calcium.

No doubt because of the lack of a suitable method, very little effort seems to have been made to determine the effect of calcium deficiency on spermatozoa, although Tyler mentions the fact that "the absence of (calcium) interferes with the agglutination of sea urchin sperm" (1948, p. 210). The reaction of the sea urchin sperm acrosome to egg-water (Dan, 1952), however, in which at least part of the acrosomal substance is released, accompanied by the projection of a fibrous structure, provides one clear-cut criterion for comparing the effects on the individual spermatozoan of various non-fatal experimental conditions, including calcium deficiency.

The observations presented in this paper will show that calcium deficiency prevents the acrosome reaction in response to various stimuli, while it tends to enhance the agglutinating effect of egg-water and prevents the loss of fertilizing capacity after reversal of agglutination.

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[1] This work was supported by a grant-in-aid from the Ministry of Education (Minkan Kenkyu Hi).

MATERIALS AND METHODS

Among the sea urchins easily available at Misaki, the spermatozoa of the three species of regular sea urchins, *Hemicentrotus (Strongylocentrotus) pulcherrimus, Anthocidaris (Heliocidaris) crassispina* and *Pseudocentrotus depressus,* show typical reversible agglutination when mixed with the egg-water of their respective species; the following observations were made on the gametes of these three species.

Eggs were obtained by the introduction of isotonic KCl into the body cavity. In the case of "Ca-low egg-water," the animal was rinsed with Ca-free artificial sea water and made to shed its eggs directly into this solution. The egg suspension was sometimes warmed to about 30° C. to hasten dissolution of the jelly, or the eggs were centrifuged at 2000 × gravity for 5 minutes and the empty jelly hull suspension warmed to about 50° C. for a few minutes. In every case the egg-water was filtered before use.

Control egg-water solutions were obtained either by centrifuging the jelly from an equivalent volume of eggs in sea water, or by adding sufficient isotonic $CaCl_2$ to the Ca-low egg-water to bring its calcium content to that of normal sea water.

Spermatozoa were collected "dry" as shed following KCl stimulation, or as they exuded from excised testes. Care was always taken to reduce contamination by sea water or body fluids. In most of the experiments, 1 or 2% of dry sperm were suspended in Ca-free solution or filtered sea water. Spermatozoa were always freshly suspended immediately before the addition of egg-water, unless otherwise specified.

Although many biological systems are sensitive to minute changes in calcium concentration, the sperm acrosome was found to be unexpectedly insensitive in this respect. For example, in *Pseudocentrotus,* the acrosome reaction was still suppressed in all sperm at 25% of the normal calcium concentration, only about half the acrosomes reacted at 50% calcium concentration, and response of all the acrosomes was only obtained at 67% of the calcium concentration of normal sea water. In view of this fact, no effort was made in these experiments to remove the last traces of calcium from the experimental solutions by the use of oxalate or citrate, and the term "Ca-free" has therefore been avoided in referring to egg-water solutions and sperm suspensions. However, it should be pointed out that the calcium content of these media was below the threshold required for fertilization to take place in them.

As in the first study of this series, sperm suspensions were fixed by the addition of neutralized formalin. Preparations for electron microscopy were made directly from these suspensions; washing out of the sea water salts was done after mounting on the collodion membranes. Some of the preparations were shadowed with Cr_2O_3; the electron microscope used was a Hitachi Standard, operating on 50 KV.

RESULTS

The reversible agglutination of the spermatozoa of these three sea urchin species follows the usual pattern, which has been described in detail by Jacqus Loeb (1914) for two species of *Strongylocentrotus.* While there are certain details of the agglutination reaction which seem to be peculiar to one or another of the species under consideration, description of these will be omitted, and the major aspects of the reaction common to all the species will be reported. Unless otherwise specified, the statements will apply to all three species.

Attempts to treat the agglutination reaction quantitatively run into many difficulties because of the lack of a clear end-point in the reaction itself and the virtual impossibility of standardizing the reactants (see Goldforb, 1929a, 1929b, 1929c, for a detailed discussion of individual variation among sea urchin gametes and the effect of ageing of spermatozoa on agglutination). In order to report the phenomenon with any semblance of clarity, therefore, it has seemed necessary to disregard the borderline data and concentrate attention on the cases which consistently give positive or negative results.

Spermatozoa suspended in Ca-free sea water

If spermatozoa freshly suspended in Ca-free sea water are compared under the high power of the phase contrast microscope with a similar sample in ordinary sea water, a striking difference is immediately observable between the behavior of the sperm in the two suspensions. The majority of the spermatozoa in sea water are either trapped against the glass surfaces on their sides or have undergone the acrosome reaction on contact with the glass and are rotating around their tips, held by the acrosome process (Dan, 1952). In the Ca-low preparation, on the other hand, all the spermatozoa are found to be in vigorous locomotion, describing uniformly-sized circles against the glass surfaces. When these have stopped moving sufficiently to permit close observation, it can be seen that none of them has reacted to the contact stimulus.

A comparison of Figures 1 and 2, electron micrographs of *Hemicentrotus* spermatozoa in sea water and Ca-free artificial sea water, respectively, shows that there is no immediate effect of lack of calcium on the sperm structure. Moreover, Figure 11 indicates that at least 10 minutes' exposure to a Ca-low medium does not produce any particular change in the appearance of the apical part of the spermatozoan (compare Fig. 8).

There is a marked tendency in Ca-low suspensions for the middle piece to be dislocated laterally from its normal position, and sometimes it moves completely around to the side of the head. This effect usually appears in most of the sperm after two or three minutes in a Ca-low medium (Fig. 11).[2] Such spermatozoa showed no diminution in either activity or fertilizing capacity (when used to inseminate eggs in normal sea water).

Agglutination reaction in Ca-low sea water

When Ca-low egg-water is added to a suspension of spermatozoa in Ca-free sea water, the appearance of the resulting agglutination is essentially the same as that in sea water, with the slight difference that while practically all the spermatozoa are involved in the clusters at the height of the reaction (from 5 to 30 seconds after the addition of egg-water) in sea water, a somewhat larger number of spermatozoa

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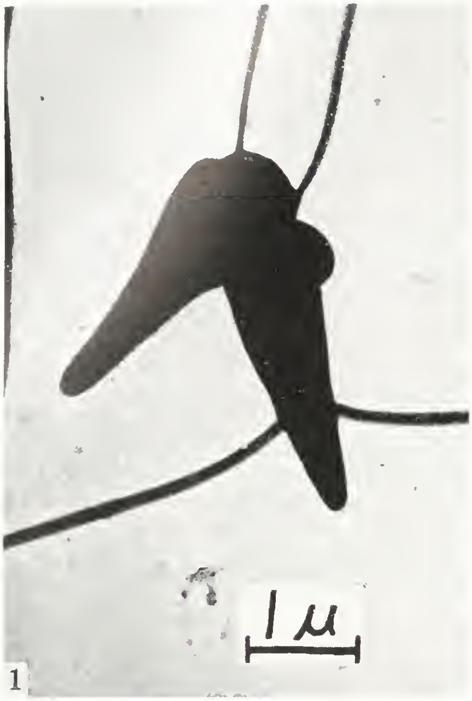
[2] In this particular case only one spermatozoan shows a slight shift in the position of the middle piece; it appears much more strikingly in Figures 10 and 12, in which the effect may have been enhanced by the addition of egg-water (see Discussion).



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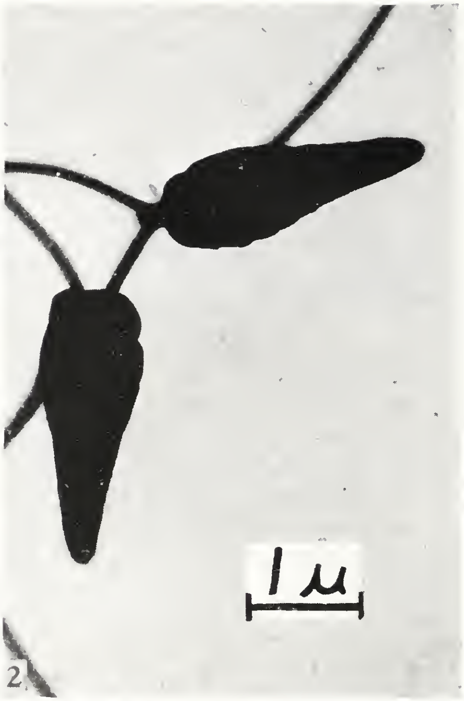

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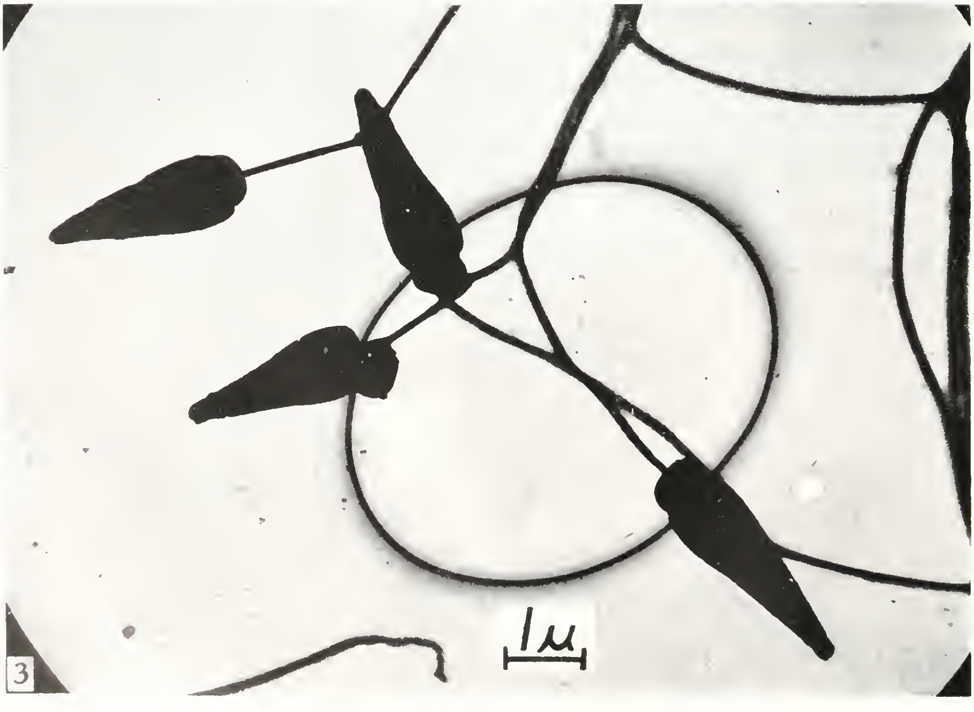

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Figs. 1–3.

can be seen swimming freely between the clumps in Ca-low suspensions. In this medium, also, the suspended clumps are strikingly regular in shape, appearing to be perfectly spherical, in contrast to the more irregular masses formed in sea water agglutination.

One of the characteristics of reversible agglutination which is measurable to some extent is the duration of clumping. In all three of the species under consideration, it was found that, in general, a longer time was required in Ca-low than in sea water suspensions for reversal of agglutination. In *Hemicentrotus* this effect was only obtained with concentrated egg-water, such that in the sea water control a residue of small, permanently agglutinated clumps was left after the bulk of the suspension had returned to a free-swimming state. In *Anthocidaris,* Ca-low agglutination lasted 3–4 times as long as in the sea water controls, and in *Pseudocentrotus,* about twice as long.

Another quantitative comparison which can be made is that the effectiveness of a given solution of egg-water falls off much more rapidly with successive dilutions in the absence of calcium. For example, a lot of Ca-low egg-water which causes just perceptible (with low power microscopic observation of 1–2 cc. of suspension in a Syracuse watch-glass) agglutination of a Ca-low sperm suspension at a dilution of 1 : 64 may, after adjustment of its calcium content to that of normal sea water, cause an approximately equal agglutination of spermatozoa suspended in sea water at a dilution of 1 : 512.

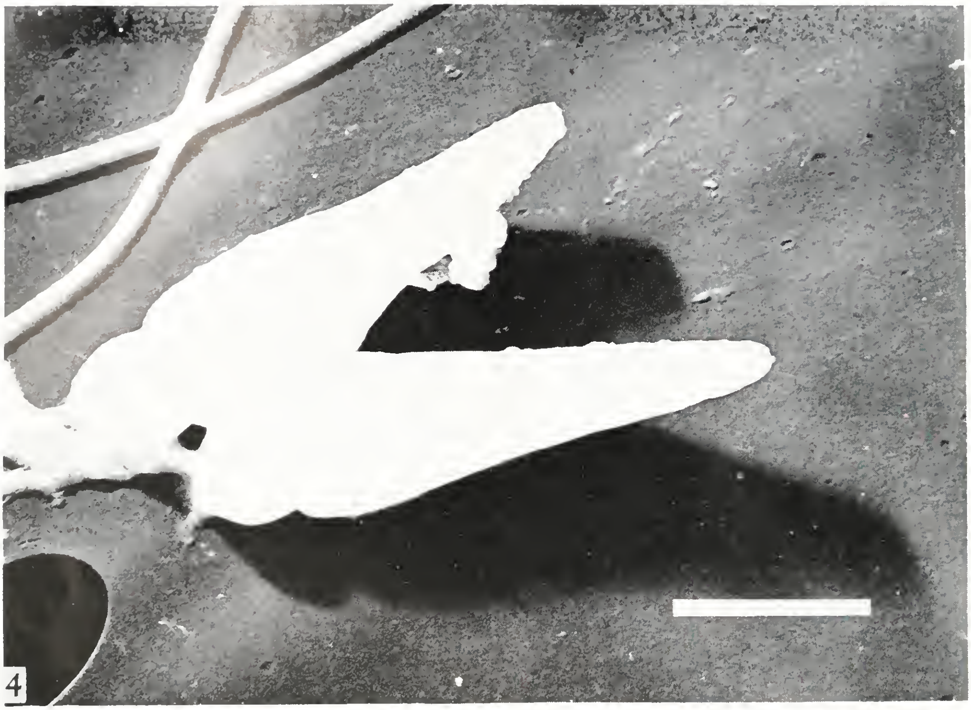
In a sample of agglutinated sperm suspension in sea water, observed between slide and cover glass with high phase contrast magnification, there are practically no free-swimming spermatozoa, since the sperm which are not involved in the clumps are found held to one of the glass surfaces by their acrosome processes. In a sample taken after reversal of agglutination, nearly all the spermatozoa become attached to the glass, and the fact that their acrosomes have reacted is clearly observable. Electron microscopical observation of such suspensions after fixation shows all the spermatozoa with acrosome processes (Dan, 1952, Fig. 1).

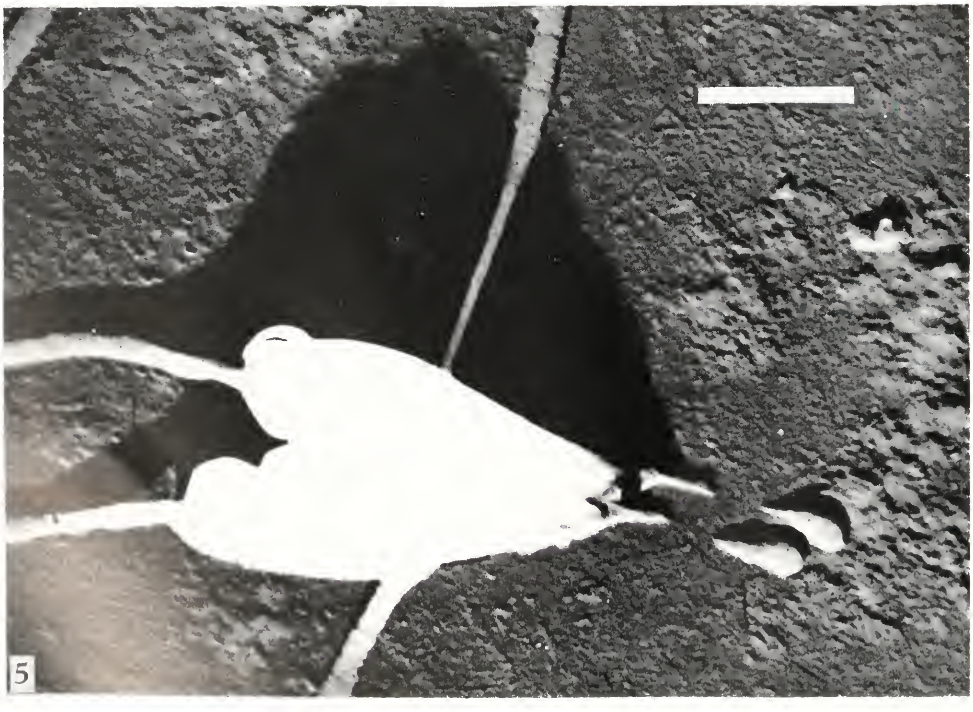
In a similar sample in Ca-free sea water, however, all the sperm which are not held in the clumps are swimming freely, exactly as in Ca-low suspension before the addition of egg-water. Continuous observation of a small clump shows that while the total number of individuals in the aggregation does not noticeably increase or decrease, there is a constant coming and going of the peripheral members of the cluster. What appears to be the same behavior on a small scale is seen in certain more or less radial groupings of a few (5–10) spermatozoa at a time, at fixed points in the microscopic field (with the focus adjusted to the underside of the cover glass) where no object can be detected to form a center for such groups. The highly active spermatozoa abruptly join the group, their heads remaining more or less stationary for a few seconds or even minutes, although their tails are

FIGURE 1. Electron micrograph of *Hemicentrotus pulcherrimus* spermatozoa, suspended for one minute in sea water and fixed with neutralized formalin.

FIGURE 2. *H. pulcherrimus* spermatozoa suspended in Ca-free artificial sea water for one minute, fixed with neutralized formalin.

FIGURE 3. Spermatozoa of *H. pulcherrimus* in Ca-free sea water, to which was added an equal amount of Ca-low egg-water. Fixed 30 seconds after the addition of egg-water, at the height of the agglutination reaction.





FIGS. 4–5.

lashing vigorously, and then they depart as suddenly as they arrived. In these small aggregates, as in the larger ones, the number of spermatozoa at any given time does not change materially. This behavior continues in the sample under the cover glass as long as the spermatozoa remain active, without respect to the reversal of agglutination in the main part of the suspension.

Acrosome reaction in Ca-low sea water

The easily observable fact that spermatozoa in a Ca-low medium do not adhere by their tips to the glass surfaces, even in the presence of egg-water, indicates that the acrosome reaction does not take place in such a medium. Phase contrast observation of individual acrosomes shows them all to be identical in appearance with those of freshly shed spermatozoa in pure sea water, and electron micrographs confirm this observation (Figs. 3, 10 and 12; compare with Figs. 1 and 8).

In order to differentiate between spermatozoa which had been involved in the clumps and those which had not, a strongly agglutinated suspension was hand-centrifuged to separate the clumped sperm from the freely swimming fraction. The sedimented clumps were re-suspended in fresh Ca-free sea water and examined for acrosome reaction. Both phase contrast and electron microscopic observation showed no difference between the acrosomes of these and of spermatozoa in the stock suspension (in Ca-low sea water before the addition of egg-water).

To control these observations, 0.36 M $CaCl_2$ was added to the Ca-low egg-water, making its calcium content equal to that of normal sea water (4%). This was then mixed with spermatozoa freshly suspended in sea water. Figures 4, 5, 6 and 7 show electron micrographs of spermatozoa of *H. pulcherrimus* fixed in pure sea water, immediately after the addition of egg-water, at the height of the agglutination reaction and after its reversal, respectively. Figures 8 and 9 show spermatozoa of *P. depressus* in sea water, and 30 seconds after the addition of egg-water.

Fertilizing capacity after agglutination in Ca-low medium

The fact has been repeatedly pointed out (Lillie, 1913; Tyler, 1941) that interaction with an agglutinating agent lowers the fertilizing capacity of a sperm suspension. The amount of diminution has been found to vary considerably from species to species and even among individuals within a given species, but in the writer's experience, also, there is always some reduction in fertilizing capacity following agglutination, when this takes place in a calcium-containing medium. It is equally clear, in the three sea urchin species under consideration, that there is no loss whatsoever in fertilizing capacity when the spermatozoa are agglutinated in Ca-low sea water. In this case, also, the clumped spermatozoa were quickly centrifuged out and re-suspended in fresh calcium-free medium, but no reduction in fertilizing capacity could be found after reversal of agglutination, either in these or in the

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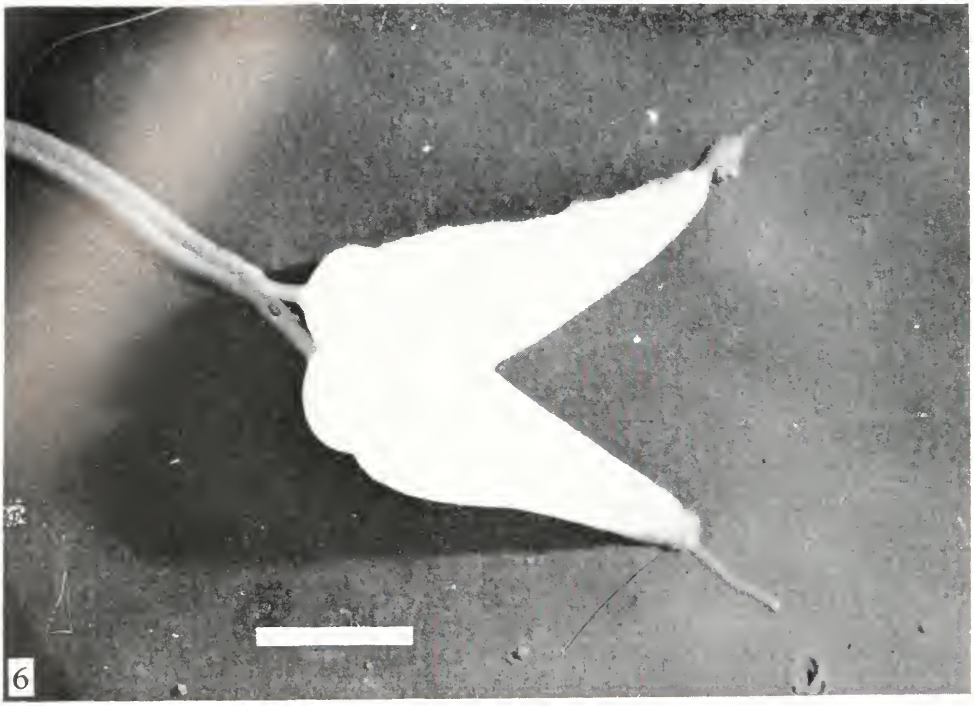
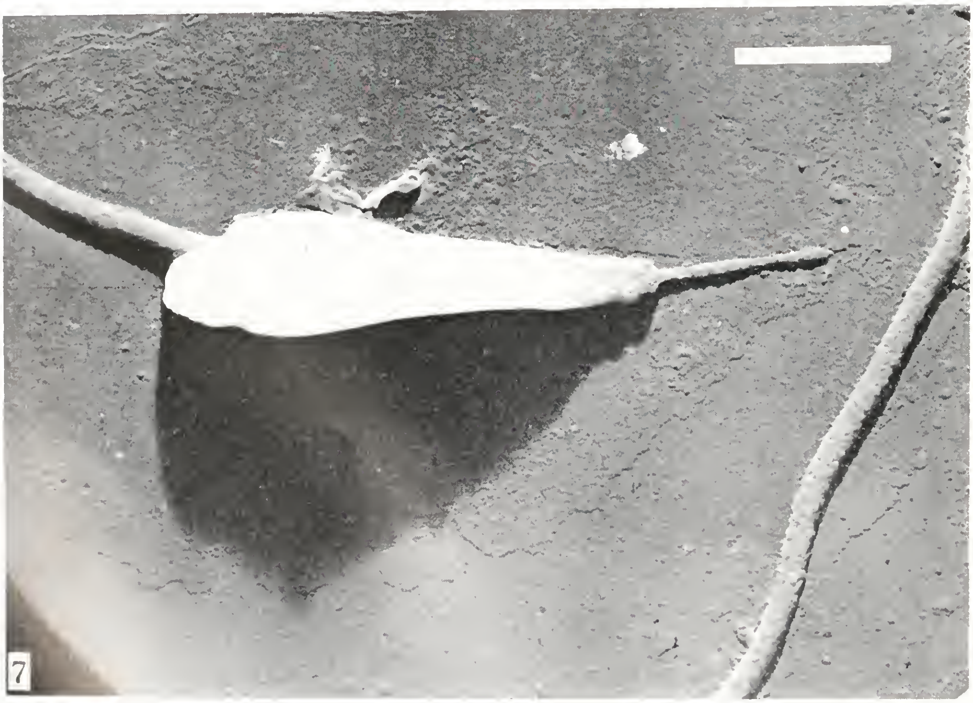
FIGURE 4. Spermatozoa of *H. pulcherrimus* in sea water, fixed with neutralized formalin. Chrome-shadowed electron micrograph.

FIGURE 5. *H. pulcherrimus.* Spermatozoa fixed two seconds after addition of homologous egg-water to sea water suspension. Breakage of acrosome filament commonly found in preparations fixed immediately after addition of egg-water (see Dan, 1952, Figs. 4 and 5).





Figs. 6–7.

free-swimming fraction of the original suspension, when they were used to inseminate eggs in sea water.

In corresponding experiments with *H. pulcherrimus* spermatozoa agglutinated in sea water, it was found that while the supernatant fraction gave between 35 and 70% fertilization, similar dilutions of the clumped-reversed sperm gave between 0 and 3%.

Discussion

These results include a number of aspects for which no explanation is forthcoming at present. On the other hand, there are certain points which stand out with unexpected clarity. The most striking of these is the failure of a typical acrosome reaction when sea urchin spermatozoa are reversibly agglutinated by homologous egg-water in the virtual absence of calcium ions. This complete separation of the two reactions, which are normally elicited simultaneously by the one stimulus of egg-water, calls for a reconsideration of the problem in more detail.

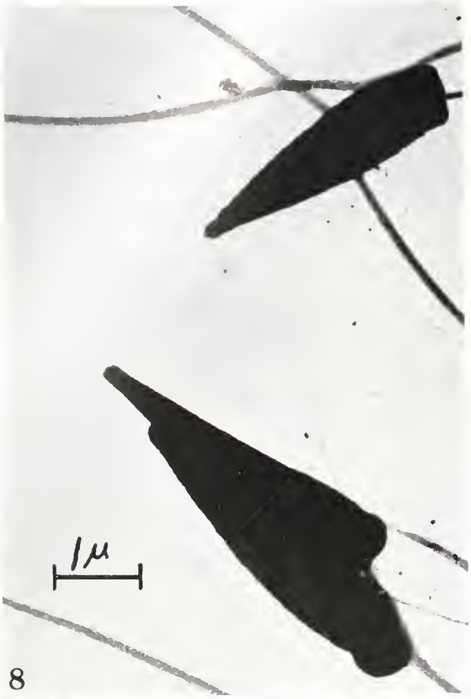
An acrosome reaction to the stimulus of egg-water, which he thought provided an explanation for the reversible agglutination phenomenon, was first reported by Popa in 1927. Using Janus green-stained Arabacia sperm, Popa found that a sticky substance was produced at the tips of the sperm heads. He believed that this substance held the sperm together in clusters, and that its dispersion accounted for the reversal of agglutination. Improved optical apparatus has shown, however, that the substance (or structure) extruded from the sperm acrosome has apparently the same shape and adhesive properties long after the agglutination clusters have broken up (Dan, 1952). Popa's idea thus loses a good deal of its plausibility, since it fails to explain reversibility.

There are, however, more fundamental faults to be found in this scheme, since close observation of reversibly agglutinated spermatozoa clearly indicates, as Jacques Loeb (1914) reported forty years ago (p. 127), " . . . that the spermatozoa at the periphery of a cluster are in free progressive motion. When the clusters were small or when the sperm suspension was thin it was possible to observe the spermatozoa which are in the center of the cluster. It was seen that the spermatozoa in the center also were in very lively motion, with the possible exception of small lumps or groups of spermatozoa which may have stuck together. The clusters reminded the writer of a dense swarm of insects which move like a coherent mass through space. These clusters move like one solid body through the water, notwithstanding the fact that the individual spermatozoa are free to scatter."

This easily observable freedom of movement on the part of the agglutinated spermatozoa, as well as the fact that the regular clusters begin to be formed the moment egg-water is introduced into a sperm suspension and attain a large size within a few seconds by the fusion of smaller clumps, almost certainly rules out any possibility that the acrosome reaction can provide a physical basis for re-

FIGURE 6. *H. pulcherrimus* spermatozoa fixed 20 seconds after acrosome reaction. The substance which was extruded has dissipated, exposing the acrosome filament.

FIGURE 7. *H. pulcherrimus* spermatozoan fixed three minutes after addition of egg-water, 30 seconds after reversal of agglutination. The acrosome filament has the same appearance as in the previous sample, fixed at the height of agglutination.



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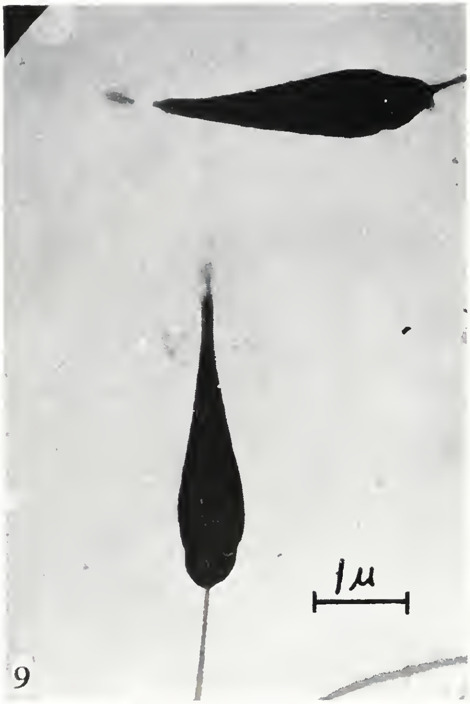

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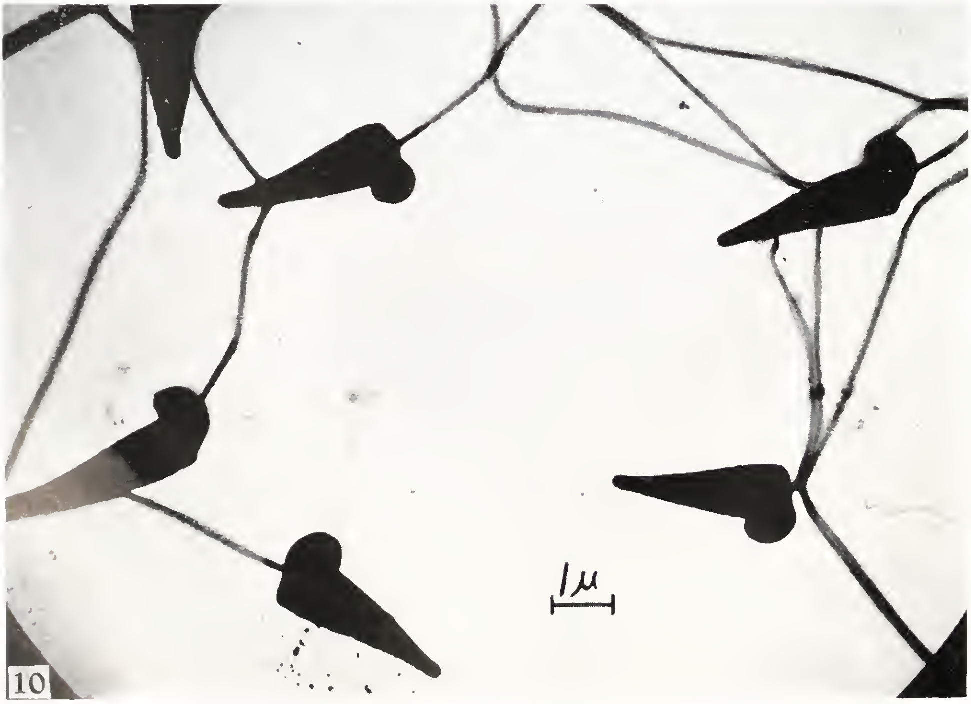

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Figs. 8–10.

versible agglutination. There is obviously some other force which instantaneously orients the swimming spermatozoa into groups and holds them there for a predetermined period, at the end of which they again become free to resume their independent behavior. Whether this is a tropistic reaction, as Loeb rather doubtfully suggested, a chemical reaction between fertilizin molecules and antifertilizin on the sperm head, as Tyler proposes, or some entirely different sort of effect, the writer does not know.

This negative conclusion, that the acrosome reaction cannot provide a causal explanation of the regular, oriented clustering found in reversible agglutination, is emphasized by comparison with the process of irreversible agglutination which follows exposure to hyperalkaline sea water, some foreign egg-waters, and various serum- or other protein-containing solutions. In these media the entire spermatozoan becomes sticky, and the cells adhere to each other at random, the whole suspension gradually becoming immobilized. As Loeb has described it (1914): ". . . the spermatozoa at first stick together to form short rows or threads; and later the threads begin to stick together to form irregular networks. At no time is there any appearance of cluster formation."

It is quite conceivable that small, regular clusters might be formed if each acrosome should become only sticky enough to adhere to another sticky acrosome,[3] but the time required for such cluster formation would greatly exceed the one or two seconds within which cluster formation is achieved in these species, and such an explanation does not provide at all for the addition of the second and succeeding layers of spermatozoa, or for the fusion of small clusters to form large ones.

A second kind of evidence dissociating agglutination and the acrosome reaction is found in the fact that sea urchin acrosomes react to the non-specific stimuli of contact with solid surfaces and hyperalkalinity, in neither of which reversible agglutination is involved.

In the third place, there is the observation presented in this paper, that the minimum calcium requirement for reversible agglutination is very much lower than that for the acrosome reaction, so that it is possible to secure a typical agglutination reaction without any detectable response of the acrosomes.

Summarizing this evidence against a causal relation between the two reactions, we find that:

1. reversibly agglutinated spermatozoa are freely moving within the clusters, and therefore cannot be considered to be held together by any substance released from the acrosome;
2. there is no significant difference, in either living or fixed sperm, between the appearance of the acrosomes at the height of agglutination and after reversal;

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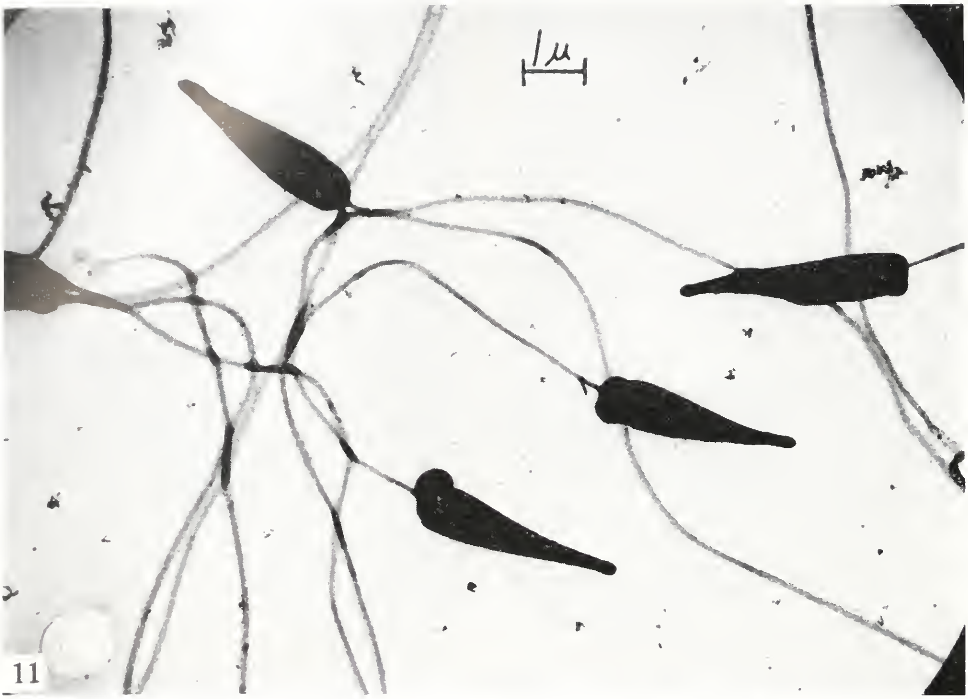
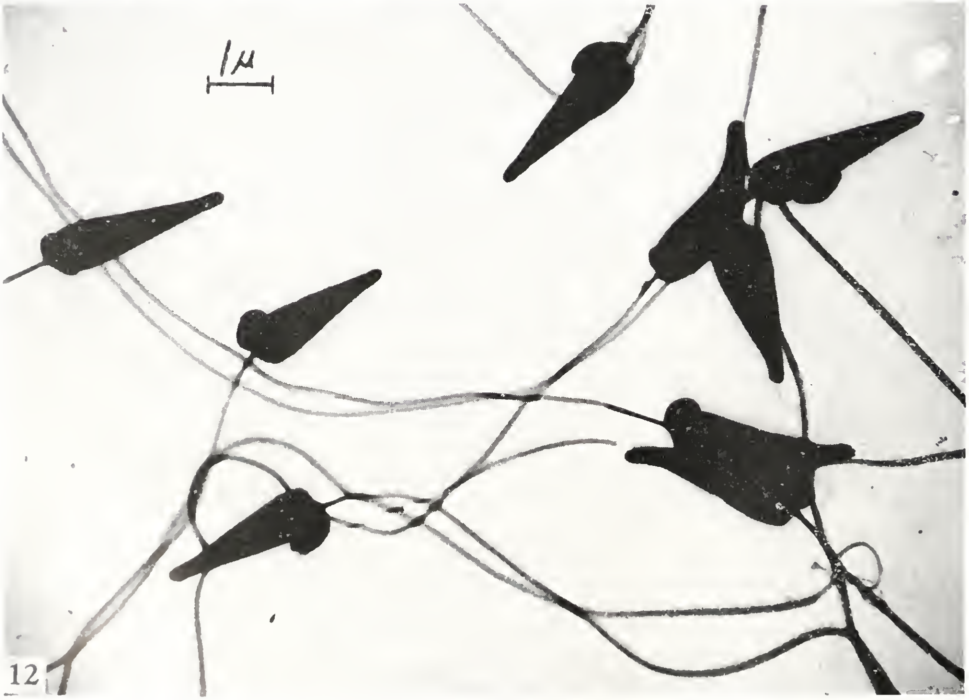
[3] This may be the explanation of "rosettes" which are formed by the sperm of some echinoderms (*e.g., Mespilia globulus*) at the air-water and water-glass interfaces as a response to homologous egg-water.

FIGURE 8. Spermatozoa of *Pseudocentrotus depressus,* fixed after one minute in sea water.

FIGURE 9. Acrosome reaction of *P. depressus,* in response to homologous egg-water.

FIGURE 10. Spermatozoa of *P. depressus* suspended in sea water, and fixed 30 seconds after addition of Ca-low egg-water. Note lateral shift of middle pieces; this condition is also usually found in Ca-free suspensions before addition of egg-water.





Figs. 11–12.

3. the clusters appear almost instantaneously after the addition of egg-water, which seems to preclude the possibility that they are formed by random acrosome-to-acrosome collisions. Moreover, this explanation would include only the smallest clusters;
4. the acrosomes react to stimuli which do not cause reversible agglutination, such as contact with solid objects and hyperalkalinity;
5. in the virtual absence of calcium, the stimulus of egg-water causes agglutination without any acrosome reaction.

Another, and perhaps more important, fact which emerges from the results of these experiments is that reversible agglutination without acrosome reaction has no effect on the fertilizing capacity of the spermatozoa. Lillie (1913) first showed that sperm suspensions which had been agglutinated by fertilizin had undergone a considerable (ca. 50%) reduction in fertilizing capacity. Tyler (1941), repeating Lillie's work, found a much greater loss of fertilizing capacity (p. 196): "Insemination with amounts of sperm that were well above the control minimum for 100 per cent fertilization gave in all tests with the agglutinated and reversed sperm between 0 and 3 per cent fertilization."[4] Rothschild (1947) has suggested that reversible agglutination may be (p. 241) "a form of false fertilization between spermatozoa instead of between eggs and spermatozoa; or at any rate, that there is some reaction between the surfaces of the spermatozoa which is sufficiently similar to that which occurs between an egg and a spermatozoon to alter the spermatozoon in an irreversible manner." Spermatozoa which have thus lost their fertilizing capacity following agglutination, Rothschild has characterized as being "muzzled" as the result of exposure to fertilizin in solution.

Among the three Misaki sea urchin species which undergo reversible agglutination, the writer has found considerable variation in the fertilizing capacity of reversed spermatozoa, especially if they are used for insemination immediately after reversal of agglutination. However, in every case there is a significant reduction in fertilizing capacity after agglutination in normal sea water, in which a majority of the acrosomes have reacted, and no reduction at all when agglutination has taken place in a calcium-low medium, where none of the acrosomes has reacted.

This result strongly suggests a causal linkage between the acrosome reaction and the fertilization reaction. Within a strict time limit, it appears that suspensions of spermatozoa with intact acrosomes possess full fertilizing capacity, while in those in which the acrosomes have reacted to such a stimulus as egg-water, this capacity is reduced. The suggestion fairly makes itself, that this acrosome reaction in response to fertilizin is the "false fertilization" of Rothschild, and its completion at a distance from the egg surface constitutes the muzzling effect on the spermatozoa.

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[4] In these experiments, Tyler used very strong egg water, such that "agglutination usually lasted 30–40 minutes" (loc. cit.). Loeb (1914) gives 2–10 minutes as the range of duration of the agglutination reaction in the species used by Tyler (*Strongylocentrotus purpuratus*); the Misaki species also fall within this range. The possibility should be considered that egg-water strong enough to cause such extreme agglutination may have had a toxic effect on the spermatozoa.

FIGURE 11. Spermatozoa of *P. depressus* fixed 10 minutes after suspension in Ca-free sea water.

FIGURE 12. Spermatozoa of *Anthocidaris crassispina* suspended in Ca-free sea water and fixed one minute after addition of Ca-low egg-water, at height of agglutination reaction.

The studies of Yamamoto (on fish eggs) and of Moser, cited above, have shown that the series of linked reactions constituting activation, the response of the egg, breaks down when calcium is excluded from the medium. The observations presented in this paper can be taken to indicate, however, that the stimulus itself is lacking, because of the failure of the acrosome reaction, when insemination takes place in a calcium-free medium.

Reference was made earlier to the change in position of the middle piece, which often occurs in all spermatozoa after a short exposure to plain Ca-low medium, without causing any diminution in the fertilizing capacity of the suspension. This same effect has been reported by Tyler (1952), who observed it in spermatozoa, suspended in sea water, which had been exposed to homologous egg-water. Tyler interprets this as an evidence of a general loosening of the sperm structure, preparatory to the complete separation of its parts within the egg cytoplasm. In this connection may be mentioned a similar relaxation of tension which occurs in starfish spermatozoa at the time of the acrosome reaction (Dan, 1954).

It is, moreover, almost certainly this same shifting of the middle piece under the influence of egg-water and various vital stains which was observed by Popa (1927) in *Arbacia* spermatozoa, although he described it as the formation of a "lateral body." However, his drawings show the middle piece tending to move around the basal end of the sperm head, and, significantly, his final figure representing the extruded "lateral body" at the side of the nucleus, shows a sperm head without any (other) middle piece.

ACKNOWLEDGMENT

It is a pleasure to acknowledge again the willing cooperation of the staff of the Misaki Marine Biological Station, and the kindness of Prof. A. Takamiya, of Tokyo Institute of Technology, who arranged for the use of the Institute's electron microscope. The photography was done by Mr. H. Akabori.

SUMMARY

1. When sea urchin spermatozoa are suspended in Ca-free artificial sea water,

a) they show no morphological change, except that there is a tendency for the middle piece to become "loose" and move around to the side of the nucleus;
b) the sperm swim vigorously and attack the surface of unfertilized eggs as in normal sea water, but without being able to penetrate;
c) they do not undergo the acrosome reaction on contact with glass or collodion surfaces.

2. If "Ca-free" egg-water is added to such a sperm suspension,

a) a regular, reversible agglutination reaction occurs, which, with strong egg-water, lasts 2–4 times longer than in a corresponding suspension in which the calcium content is adjusted to that of normal sea water;

b) no acrosome reaction can be detected in spermatozoa thus agglutinated in the virtual absence of calcium;

c) after reversal of agglutination, such spermatozoa show no reduction in fertilizing capacity when used to inseminate unfertilized eggs in sea water. This is in marked contrast to the sea water control, in which there is a significant loss in fertilizing capacity following agglutination.

3. It is concluded that:

a) the agglutination reaction and the acrosome reaction must be considered to be separate phenomena, both occurring in response to the stimulus of egg-water, but not causally interrelated;

b) in view of the fact that fertilizing capacity is retained to the full extent by sperm suspensions in which the acrosomes remain intact, and is at least partially lost following mass acrosome reaction, this acrosome reaction must play an important role in the process of fertilization.

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ACTION OF AQUEOUS EXTRACT OF BEEF SPLEEN ON CELLS OF SARCOMA 37 ASCITES[1]

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An inhibitory action of beef spleen extract on growth of sarcoma 180 and mammary carcinoma of the mouse has been reported by Lewisohn (1938, 1945); of mouse sarcoma 37 and methylcholanthrene-induced sarcomas by Diller and Watson (1949); and of Brown-Pearce carcinoma of the rabbit by Nuttini *et al.* (1951). The mouse tumors studied by Diller and Watson (1949) showed degenerative cell changes resulting in shrunken bean-shaped nuclei and vacuolated cytoplasm at certain concentrations and dose levels, but no damage could be detected in normal tissues of the treated mice. MacFarlane and collaborators (1948) reported similar degenerative changes when cells of certain spontaneous tumors were stored in concentrated aqueous spleen extract for varying lengths of time at 5° C., which led them to believe that spleen extract could have a direct cytolytic action. More recently Katzberg (1952) incubated fragments of sarcoma 180 and of normal mouse tissues at 37° C. in Parker's medium and in medium containing either Rockland mouse spleen extract or Rockland mouse spleen pulp. Both malignant and non-malignant tissues remained viable in Parker's medium for ten days or longer. Splenic preparations apparently produced an agent which accelerated cytolysis. Maximum sensitivity to this agent was shown by sarcoma 180 (total destruction of cells in 48 hours) and maximum resistance by normal tissues of the Rockland mouse.

Our experiments (1949) with trocar-implanted or chemically induced tumors indicated that regression was accompanied by a marked inflammatory reaction in the vicinity of the tumor and an upsurge of mitotic activity in the organs of the hemopoietic system; a direct destructive action could not, therefore, be ascribed to the splenic extracts which we employed. Neither do such *in vitro* experiments as those of Katzberg (1952) offer clear-cut proof of direct cytolytic action, since under the conditions of his experiments, untreated tumor tissues, as well as normal tissue controls, were undergoing degeneration from the outset, and the effect of the extract, though more rapid in the case of tumor tissue, was largely an acceleration of degenerative change. The most convincing evidence of direct effect in Katzberg's experiments was the occurrence of metaphase block in sarcoma 180 during the first 24 hours after exposure to his splenic preparations.

In the hope that additional light could be thrown on the action of spleen extract in direct contact with tumor cells, further experiments were carried out in this laboratory using the ascites form of sarcoma 37, the solid form of which had been studied previously in this connection (Diller and Watson, 1949).

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Material and Methods

Albino mice, 6 to 8 weeks of age, both males and females, of the special Swiss strain maintained in the main colony of our Institute were inoculated intraperitoneally with one cc. of sarcoma 37 ascites tumor. The growth was allowed to develop for three days, when abdominal swelling could be observed visually. A sample of the ascites fluid (see Fig. 1) was withdrawn, diluted 1 : 200 in saline solution and counted in a hemacytometer. The animals were then injected intraperitoneally with a single dose (one cc.) or with 4 doses (0.25 cc. each) of varying concentrations (50 mg., 100 mg., and 150 mg. of solids/ml.) of crude aqueous cell-free, beef spleen extracts (not commercially available) obtained through the courtesy of the Eli Lilly Company, or prepared at the Kitchener Clinic and processed in Philadelphia by the McNeil Laboratories.[3] In the Kitchener Clinic the extract is prepared by mincing 400 grams of fresh calves' spleen under sterile conditions and extracting it in 1000 cc. of sterile saline at 33°–35° C. Approximately 400 cc. of the supernatant is decanted after four weeks and passed through a Seitz filter. Details of preparation and clinical use will be treated in a forthcoming paper under the authorship of the medical member of the group (G. F. W.). Control animals received corresponding amounts of sterile salt solution. The extracts were found to be very unstable, even though prepared and maintained constantly at temperatures just above freezing. Color change from red to dark brown usually accompanied loss of potency.

Ascitic fluid was withdrawn from each mouse at intervals of 6, 24 and 48 hours after spleen extract injection for hemacytometer counts. Similar counts were made of control mice that had received neither spleen extract nor saline solution, in order to determine whether the cell population was affected by repeated withdrawals. This was apparently not the case, since the cell count remained essentially static. Permanent preparations of each sample were fixed in Allen's or Carnoy's fluid and stained with Feulgen-fast green for differential counts. Detailed phase contrast studies were made also of living cells stained with neutral red to aid in detection of degenerating nuclei, after the method of Goldie and Felix (1951).

Observations

Cell count. The most striking result was the decrease in tumor cell population following spleen extract injection (see Table I). This was accompanied by subsidence of the ascites swelling. Stained smears of the residual fluid (usually less than 0.5 cc. after 48 hours) revealed small clumps or islands of cells as indicated in Figure 3, which presumably served as centers for formation of solid masses. Some control animals also developed solid tumors or sheets of tumor cells on the peritoneum, but there was never any regression of the ascites swelling in untreated mice.

The reduction in number of tumor cells observed in our experiments was apparently in part the consequence of infiltration of leucocytes and histiocytes, by which the tumor cells were phagocytized, in part of cessation of mitosis, and in part of cellular degeneration (see table of differential counts).

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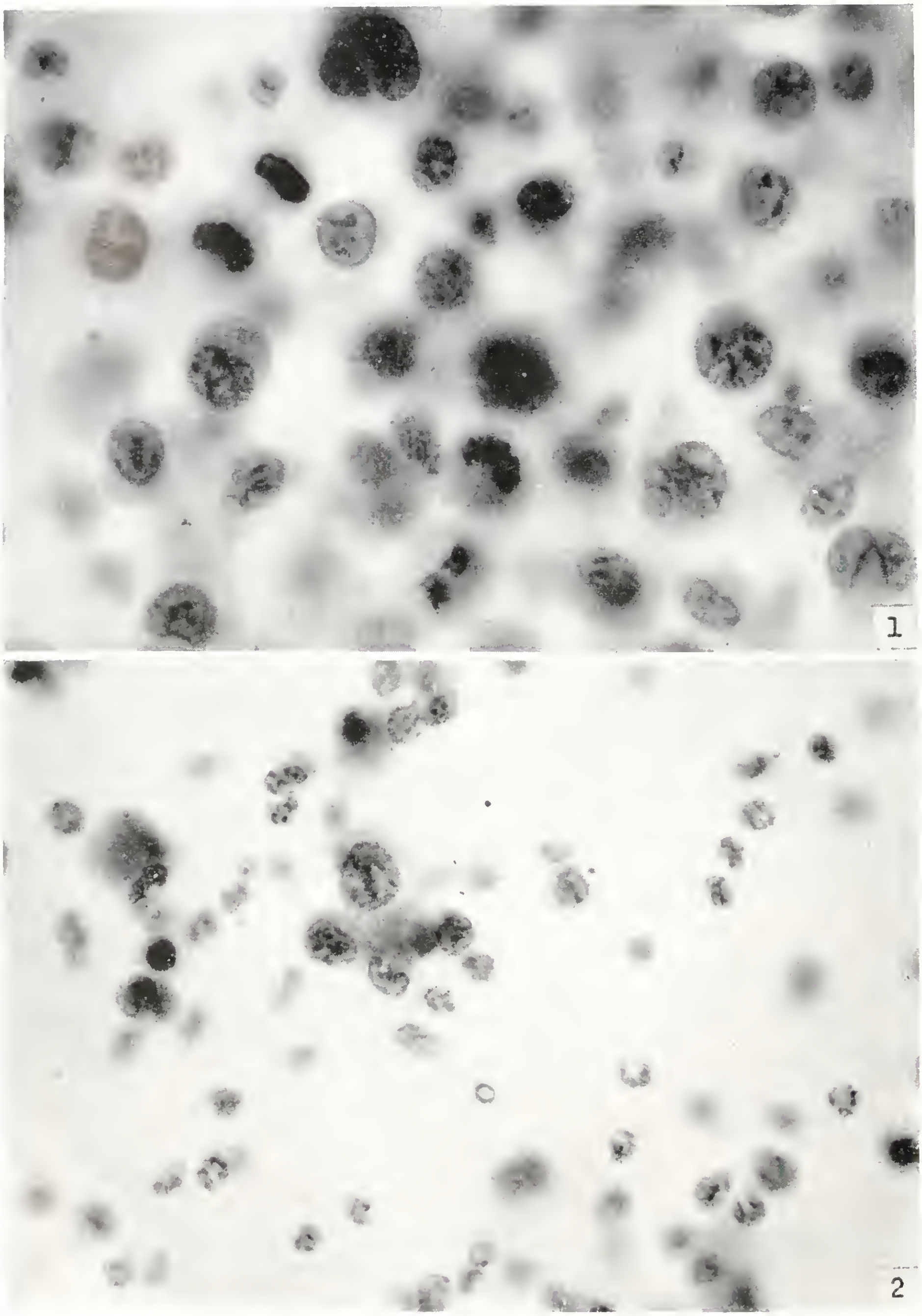
[3] The authors wish to thank Dr. Paul N. Harris of the Eli Lilly Company and Mr. Robert McNeil, Jr., of the McNeil Laboratories, for their interest and assistance.



Figs. 1–2.

Sarcoma 37 ascites tumor preparations from mice injected with aqueous beef spleen extract. Picric-formol acetic fixation, Feulgen-fast green.

TABLE I

Cell count per cc. of sarcoma 37 ascites fluid (Average of 20 animals)

	Spleen inj. One cc. of 150 mg. conc.	Saline inj. One cc.	Uninjected
Pre-inj.	50.2×10^6	54.0×10^6	54.9×10^6
6 hrs. post-inject.	30.2×10^6	53.5×10^6	54.5×10^6
24 hrs. post-inject.	21.2×10^6	50.3×10^6	53.7×10^6
48 hrs. post-inject.	26.0×10^6	51.7×10^6	51.2×10^6

Mitotic phenomena. Counts of 1000 cells per specimen were made on stained preparations of fluid withdrawn prior to spleen extract injection, and at 6 hours, 24 hours, 48 hours and 72 hours post-injection. An average of 3.6% of the tumor cells were in mitosis just before treatment. In many individuals no mitotic figures at all could be observed 6–24 hours after injection of spleen extract, and in all individuals there was a decreased percentage of cells in mitosis (see Table II). When one cc. of spleen extract (150 mg. of solid/cc.) was administered in a single dose, mitotic activity was suppressed for at least 48 hours, but if smaller doses of extract were given, it was necessary to repeat them at 6-hour intervals in order to achieve this result.

TABLE II

Differential count, based on 1000 cells per mouse per sample*

	Mitosis %				Necrosis %			Polymorphs %				Lymphs %				Tumor cells (non-dividing) %			
Treatment:	Pre	6 h	24 h	48 h	Pre	6 h	48 h	Pre	6 h	24 h	48 h	Pre	6 h	24 h	48 h	Pre	6 h	24 h	48 h
Spleen extract 50 mg./ml.	3.1	0.3	0.0	0.2	0.4	1.5	3.8	17.5	39.8	59.7	32.4	3.0	10.0	13.6	24.0	76.0	48.4	26.7	39.6
Spleen extract 150 mg./ml.	3.7	1.4	1.4	0.1	0.4	0.4	60.1	20.9	28.8	40.0	24.9	4.4	4.3	6.0	9.4	70.6	65.1	52.6	5.5
Saline inj.	3.9	3.5	3.6	3.7	1.1	0.2	1.5	20.3	16.8	18.1	19.4	8.5	7.5	9.7	6.1	66.2	72.0	68.6	69.3
Non-inj. controls	3.6	3.4	3.8	3.8	1.3	1.2	2.9	13.0	13.9	18.9	16.2	9.5	10.2	9.9	10.8	72.6	71.3	68.3	66.3

* 20 mice in each group.

Blood cell counts.

a. *In peritoneal fluid.* The number of polymorphonuclear leucocytes increased immediately after spleen injection in all cases (Fig. 2), and continued to rise for about 30 hours, then gradually returned to the level of the controls as shown in Table II. There was an accompanying increase in percentage of lymphocytes, which persisted into the 48-hour period. The lymphocytic count agrees with that

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FIGURE 1. Smear preparation of untreated control, three days after inoculation into the peritoneal cavity. Ca. 800 ×.

FIGURE 2. Smear preparation of ascitic fluid withdrawn from the same mouse 24 hours after intraperitoneal injection of spleen extract (50 mg./ml.). Only two tumor cells remain in a comparable field; the remainder of the cell population is made up of leucocytes. Ca. 800 ×.

observed when solid tumors were treated with spleen extract as described in an earlier paper (Diller and Watson, 1949). Macrophages and other histiocytes were present also in the ascitic fluid, but there was no significant change in number of these cells following spleen injection, as was the case with solid tumors (Diller and Watson, 1949; Lewisohn, 1938, 1945).

b. *Peripheral blood.* A comparison of the effect of injection of spleen extract on the peripheral blood picture (tail vein samples) of tumor-bearing and non-tumor-bearing mice appears in Table III.

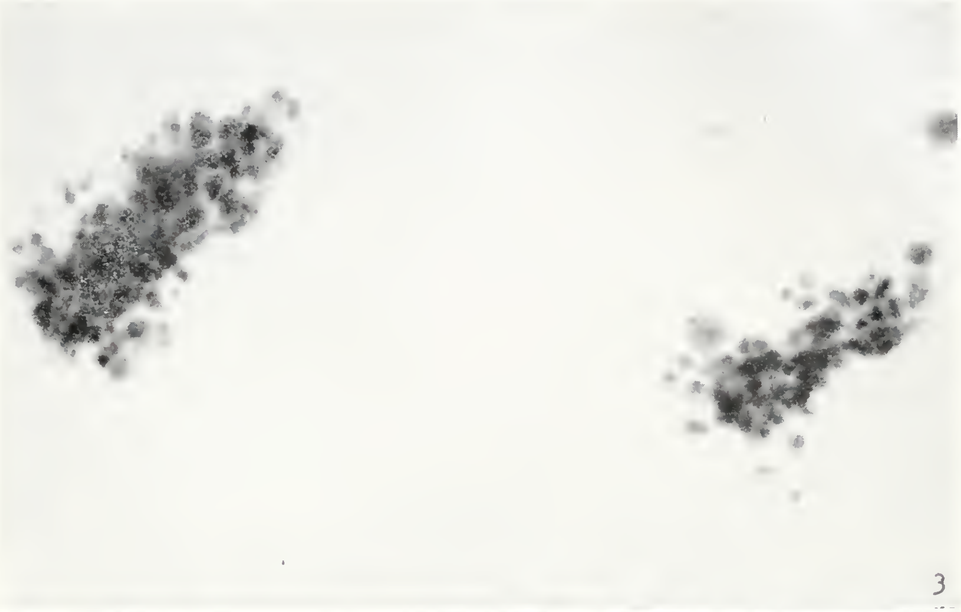
TABLE III

Peripheral (tail vein) leucocyte count (Average of 20 mice)

Mouse strain	Tumor- or non-tumor-bearing	Treatment	Route of inj.	Duration of treatment	W.B.C. count/mm.^{3} 1:20 dil.
Swiss	Non-tumor	—	—	—	15,459
Swiss	Non-tumor	Spleen ext. 150 mg./cc. 1 cc. daily	I.P.	6 days	15,740
Swiss	S-37 solid 7 days post-implant.	—	—	—	21,990*
Swiss	S-37 solid 7 days post-implant	Spleen ext. 100 mg./cc. 1 cc. daily beg. 4th day post-implant.	I.P.	24 hrs. 72 hrs.	30,060 32,530
Swiss	S-37 ascites 5 days post-implant.	—	—	—	18,500
	S-37 ascites 5 days post-implant.	Spleen ext. 150 mg./cc. 1 cc. on 3rd day post-implant.	I.P.	48 hrs.	39,120

* Data from an earlier experiment (1949). Fekete in *Biology of the Laboratory Mouse*, 1941, p. 94, reported an average peripheral count (based on unpublished data of Law and Heston) of 21,510 for tumor-bearing Bagg albinos.

Percentage of viable tumor cells. The percentage of non-dividing tumor cells before and after injection, based on a count of 1000 cells per preparation, is also shown in Table II. Animals injected with the Lilly extract showed an average decrease in number of tumor cells from 76% before injection to 26.7% at 24 hours. This was apparently due to several factors—dispersion of cells by reason of infiltration of leucocytes, cessation of mitosis, and, as indicated by uninjected controls, to slight lowering of cell numbers by daily withdrawal of fluid. With the fall of the leucocyte count at 48 hours and resumption of mitosis, the tumor cell count per 1000 cells was increased to 39.6%. Following the injection of an extract



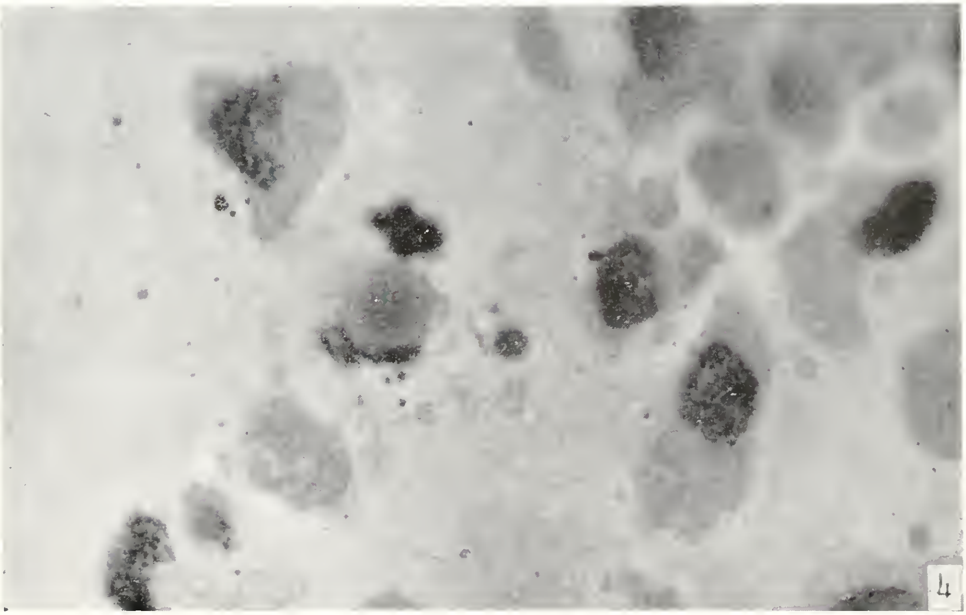


Figure 3. Smear preparation of ascitic fluid from the same mouse 72 hours after intraperitoneal injection of spleen extract. Small solid tumors are beginning to form around islands made up of aggregations of tumor cells and leucocytes. Ca. 400 ×.

Figure 4. Smear preparation of ascitic fluid from a mouse 48 hours after intraperitoneal injection of spleen extract (150 mg./ml.) showing total degeneration of almost all tumor cells. (In such cases only about 5% of the tumor cells remained viable.) Ca. 1100 ×.

containing approximately 150 mg. of solids ml., the percentage of apparently viable tumor cells remained very low at 48 hours (only 5.5% had a normal appearance; see Fig. 4), since most of the cells were shrunken and coagulated.

Morphological changes. The degenerating tumor cells just mentioned showed cytological changes consisting of nuclear shrinkage and pycnosis and vacuolization of the cytoplasm. These changes are essentially the same as those observed in solid tumors on the third day after beginning of spleen injection (Diller and Watson, 1949) and those reported by MacFarlane (1948) after storage of tumor cells in concentrated spleen extract. No evidence of interference with the spindle mechanism was observed in our experiments with ascites tumors, though Katzberg (1952) reported this phenomenon as occurring in the first 24 hours of storage in his splenic preparations, and the percentage of multipolarity was increased in solid S-37 treated by us with low concentrations (Diller and Watson, 1949).

Survival. Maximum survival time was attained by injecting 0.25 cc. of the Lilly extract four times daily at 6-hour intervals, beginning either immediately after inoculation of the host with ascites tumor, or on the third day post-inoculation. This induced regression of the ascites swelling in 16 out of 60 animals treated, though solid masses persisted in the abdominal cavities of these mice. Large single masses were not noticeably affected by continued spleen extract injection, but grew like a trocar-introduced solid tumor, resulting in death of 10 of the 16 solid-tumor-bearing animals in from 18–24 days (mean survival 20.7 days). Control animals bearing untreated ascites tumors died 7–9 days (mean 7.1 days) after inoculation. In the group of 16 mice in which solid tumors developed, complete resorption of smaller masses was induced in 6 animals under continued spleen therapy. The host animals were still tumor-free six months later, when they were sacrificed. It will thus be seen that even the most effective samples of extract with respect to mitotic inhibition and tumor cell destruction did not improve the chance of survival of the host unless the ascitic fluid was absorbed and the neoplasm converted to the solid form. Of these solid tumors, about one-third regressed under continued spleen extract injection. Since the fate of small solid sarcoma 37 in mice treated with similar spleen extracts is already known (Diller and Watson, 1949) further studies on larger numbers of animals were not carried out.

In most of our experiments ascites swelling was already visible when treatment was begun. This procedure was followed in order to ensure actual presence of neoplasia. However, studies were made also on the effect of injecting the extract prior to inoculation with ascites fluid. If no further spleen extract injections were made after inoculation of the tumor, the course of development was unaffected. On the other hand, when injection of the extract was resumed on the day of inoculation of the tumor, all the animals died with hemorrhage in the peritoneal cavity before ascites swelling appeared. Non-tumor-bearing control mice suffered no casualties when subjected at the same time intervals to interrupted dosage of the same magnitude. Simultaneous injection of extract into the peritoneal cavity of mice at the time of tumor inoculation did not prevent the development of the ascites tumor.

DISCUSSION

There are two types of reaction to injection of the splenic extract into mice bearing sarcoma 37 ascites:

1. *An inflammatory one* involving a sharp increase in the number of polymorphonuclear leucocytes (Fig. 2) and a less marked, but more persistent, increase in the number of lymphocytes present in the ascitic fluid. The same phenomena were observed in the case of mice bearing solid sarcoma 37 (Diller and Watson, 1949), since on the third day after beginning of treatment there was considerable infiltration of the tumor tissue by leucocytes, including an increased number of macrophages.

2. *Inhibition of mitosis* in all of the tumors and varying amounts of necrotic change (depending on the sample of spleen extract employed) with consequent reduction in tumor cell population accompanied by formation of small solid tumors.

In addition to reports of spleen-extract-induced regression of already developed tumors, noted at the beginning of this article, there have been numerous accounts of the inhibitory effect of spleen extract on establishment of tumor transplants or on experimental tumor induction. For example, Sugiura (1938) found that aqueous extracts of several organs of the rat had a depressant action on growth of mouse sarcoma 180 and on Flexner-Jobling rat carcinoma when, prior to implantation, the tumor fragments were stored in the extracts at 4–5° C. before implantation into mice. He reported also cessation of mitosis *in vitro* with degenerative nuclear changes under the same experimental conditions.

Yun (1950a, 1950b, 1950c) reported that pre-injection of mice with splenic extract inhibited development of tumors in mice painted with methylcholanthrene and also that extirpation of the spleen from A strain mice furthered the development of methylcholanthrene tumors.

When Nuttini (1951) pre-injected rabbits with rabbit spleen pulp or cell-free filtrate thereof, 66% of the host animals were protected against development of Brown-Pearce carcinoma. Therapeutic administration of the filtrate also induced total regression in a significant percentage of the animals treated.

In 1952 Foley reported retardation and/or regression of lymphosarcoma in C3H mice implanted subcutaneously with cell suspensions of spleen from an alien mouse strain. Also according to Lorenz *et al.* (1953), spleen shielding and bone marrow injections effectively protect mice against x-ray induction of lymphoid tumors. When C57 Black mice were given four doses of 225 r without spleen protection 70% of the mice developed lymphoid tumors but after the same amount of irradiation in a spleen-shielded group tumors appeared in only 3% of the animals. On the basis of studies of tumor tissue and spleen tissue grown *in vitro* Pollard and Bussell (1953) showed that spleen tissue from the host asserted no destructive effect on its own tumor tissue though similar tissue from the spleen of a rat that had sloughed a tumor had a rapid destructive effect.

That the inhibition of mitotic processes is not confined to neoplastic tissue in contact with splenic extract was indicated by Fardon *et al.* (1948) who reported partial inhibition of mitosis *in vivo* in the crypts of Lieberkühn of mice injected with beef spleen extract; and Hoffman (1940) found that extract of kidney, spleen, liver and lung definitely inhibited growth of fibroblast colonies *in vitro*.

Rohdenburg and Nagy (1937) showed that *both* inhibitory and stimulatory materials are present in rabbit and human spleen. The inhibitory agent was found in the initial acetone-soluble fraction. In our earlier experiments (1949) spleen, liver, thymus, lymph nodes and, occasionally, adrenal tissues of the treated mice were stimulated to mitotic activity by doses that were capable of causing regression of solid tumors. This led us at that time to postulate that the suppressive effect on tumors was probably due, not to direct cytolysis, but to stimulation of the hemopoietic organs and the adrenal cortex. Furthermore, extract of low concentration appeared to stimulate mitosis in solid tumors, resulting in polyploid cells (as judged by numbers of nucleoli) and multipolar spindles. It is possible that these were, in fact, damaged cells which would eventually have become inviable. Bearing on this point are the findings with respect to ascites tumors injected with the 50 mg./ml. concentration (Table II) where inhibition of mitosis was obtained more rapidly with the low than with the high concentration. However, the 150 mg./ml. preparation produced drastic cell damage and eventual destruction of a much higher percentage of tumor cells (only 5.5% viable after 48 hours) though the host animals usually did not survive more than an additional day and died ahead of the controls. Non-tumor-bearing control mice tolerated injections of the same magnitude without adverse reaction, which suggests that toxic substances were being released by the degenerating cells. Klein (1951) reported that if cells are damaged before or after introduction into the peritoneal cavity, the course of events will be the same as though low cell numbers were originally injected, *i.e.,* there occurs a very intense inflammatory reaction after which tumor cells disappear completely from the peritoneal fluid, or are present in relatively low numbers and the animals die much later with solid tumors. Apparently this is what occurred in our own experiments in 10% of the animals.

Cook and collaborators (1951) attempted to determine the mechanism by which tissue extracts operate in growth processes and reported that spleen extract has an inhibiting effect on certain enzyme systems known to function in cellular respiration. Aqueous homologous cell-free spleen preparations were the most effective in stimulating oxygen uptake. Water-soluble spleen extract caused depression in various concentrations, except those from 25–10 mg./ml., which produced stimulation. In general, neoplastic tissues showed greater changes than did normal tissues.

Whether or not splenic extract exerts a direct cytolytic action on tumor cells is still not proven since the evidence from ascites tumors is that the host participates through leucocytic response. However, the experiments herein described or reviewed appear to indicate that there are substances present in splenic extract capable of suppressing mitosis for varying periods of time and of inducing cytolysis in tumors, while at the same time stimulating the hemopoietic system of the host.

SUMMARY

1. The injection of a single dose of aqueous beef spleen extract containing 50 mg.–150 mg./ml. of solid materials directly into the peritoneal cavity of mice bearing ascites sarcoma 37 resulted in inhibition of mitosis for a period of at least 48 hours, and in some cases in complete cessation of mitosis that persisted for even longer periods. Mitotic inhibition was accompanied by a marked inflammatory reaction. Cell counts per cc. of ascites fluid were lowered and the percentage of viable tumor cells decreased, partly as the result of cessation of mitosis and necrotic change, and partly through dilution with white blood cells.

2. The reduction of tumor cell population was accompanied by the aggregation of tumor cells into islands which presumably served as centers for the formation of

solid tumors. When the tumor masses thus formed were large, no regression was observed, though survival time was considerably lengthened; however, small masses were completely resorbed under continued spleen extract therapy in a third of the animals whose tumors had become solid under spleen extract injection (10% of the total number of animals treated). No reduction in ascites swelling unaccompanied by the formation of solid tumors was observed.

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THE EFFECTS OF TEMPERATURE ON THE SUCROSE THRESHOLDS OF THE TARSAL CHEMORECEPTORS OF THE FLESH FLY, SARCOPHAGA BULLATA [1]

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It has long been known that insects possess contact chemical receptors which enable them to select desirable and reject undesirable foods. Earlier studies on the locations of these receptors are reviewed by Frings and Frings (1949) and on the functions by Dethier and Chadwick (1948). One of the many problems evolved from these studies is whether stimulation of gustatory receptors is due to absorption, in which the stimulating substance penetrates the exoskeleton, or to adsorption, in which the substance adheres to the surface of the taste receptor. Dethier (1951) suggests that penetration is most important by stating that the limiting mechanism in tarsal chemoreception probably involves a two-phase system, in which highly water-soluble compounds gain access to the receptor through an aqueous phase and the larger lipoid-soluble molecules chiefly through a lipoid phase.

A likely hypothesis, if penetration is involved, would be that the higher the temperature, within limits of death, the faster the penetration and the lower the threshold. Conversely, the lower the temperature, the slower the penetration and the higher the threshold. If, on the other hand, adsorption is involved, the negative temperature coefficient, other factors being equal, would cause the threshold to be lower at lower temperatures and higher at higher temperatures. The present study was an attempt to answer this absorption-adsorption question by testing the effects of temperature on the sucrose threshold of the tarsal chemoreceptors of the flesh fly, *Sarcophaga bullata*.

MATERIALS AND METHODS

The flies were reared in the laboratory as described by Frings and Frings (1952) and Knipe and Frings (1952). Adults were kept in wire cages (30 × 30 × 30 cm.), where they were given a constant supply of water and sugar cubes. Protein was supplied to the flies in the form of skinned dead mice. A mouse was supplied every 24 hours to each cage of flies until larviposition occurred, which was within 5–7 days after the protein was first furnished. After approximately 200 larvae were obtained, the mice were withheld until a new supply of larvae was needed. This insured a constant supply of larvae and flies at regular intervals. The larvae were reared on moistened dog biscuit.

Techniques described by Frings (1947) were used in mounting the flies for testing. After they had recovered from mounting, each was allowed to consume to satiety water and 1.0 *M* sucrose solution.

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[1] Authorized for publication as Paper No. 1876 in the journal series of The Pennsylvania Agricultural Experiment Station. Part of this is included in a dissertation presented by the junior author to the Graduate Faculty of The Pennsylvania State University in partial fulfillment of the requirements for the degree of Master of Science.

The constant temperature chamber used in this study consisted of two plywood boxes, one within the other. The inside box (56 × 56 × 56 cm.) was separated from the outer one (64 × 64 × 64 cm.) by 4 cm. of insulating material. Both chambers had removable tops with glass apertures (30 × 30 cm.) situated in the center to provide necessary illumination for the inner chamber, which was easily viewed through glass doors (30 × 30 cm.) at the front of the boxes. To aid in the maintenance of desired temperatures, a suction fan was installed near the top of the inner chamber to circulate the air after it was heated by an infra-red bulb or cooled by ice. Ventilation of the chamber was controlled by a sliding glass panel made to cover an opening (6 cm. diam.) situated directly in front of the fan. A light socket was installed near the bottom of the inner chamber for the infra-red bulb and the electrical apparatus of the light and fan were operated from the outside. A slit (5 × 30 cm.) was cut along one side of the boxes for the purpose of inserting the experimental animals. This opening was covered on the outside with compressed sponge rubber with corresponding slits through which the mounted flies could be inserted. A table (56 cm. long × 15 cm. wide × 15 cm. high) was placed in the chamber to hold Syracuse watch-glasses containing the test solutions.

Much preliminary experimental testing with sucrose molarities was done before settling on the following molarities used throughout the experiment: 0.02 *M*, 0.04 *M*, 0.06 *M*, 0.08 *M*, 0.1 *M*, 0.2 *M*, 0.4 *M*, 0.6 *M*, 0.8 *M*, and 1 *M*. A fresh stock solution (1.0 *M*) was made up every four or five days and refrigerated between tests to avoid fermentation. Other molarities were made up from the stock solution every two days.

The flies were tested in the constant temperature chamber at three ranges of temperature: 19–21° C., 27–29° C., and 37–39° C. The experimental temperature for each day was picked at random in order to avoid the flies' possibly learning a particular pattern of testing. The temperatures, 27–29° C., were those of the box without the addition of heat or ice. A pan of water in the chamber assured a saturated atmosphere in the box. For temperatures of 19–21° C., two containers, one a gallon jar and the other a half-gallon jar, were filled with ice a few hours before testing and, if needed, were refilled immediately before the admission of the flies to the chamber. For temperatures of 37–39° C., a flat pan (30 × 15 × 5 cm.) of hot water, about 50° C., was placed in the chamber to increase the humidity sufficiently to keep the flies from cooling themselves, thus keeping their internal temperature the same as that of the surrounding environment. An infra-red bulb was used as the source of heat and in a matter of minutes the temperature was at 38° C. The ventilator and fan aided in maintaining an even temperature.

After the temperature of the box was set, the flies were allowed to take all the water they wanted in order to prevent a response to the water in the sucrose solution rather than to the sucrose itself. This was administered outside the chamber. Five mounted flies were tested at a time. The flies were allowed to remain in the chamber for 5–10 minutes before testing to reach the temperature of the chamber and were then tested, one at a time, first with water to make certain they had all they wanted, and then from the lowest molarity of sucrose to the highest. A rest period of one or two minutes was allowed after each few tests, so that the fly would not become fatigued to the sucrose.

Each test was made by touching the fly's tarsi to the solution in the Syracuse watch-glass. If the fly dropped its proboscis, it was considered a positive response. If the fly did not drop the proboscis at a low molarity, the fly was tested at successively higher molarities until a positive response was obtained. The flies were always tested from the low molarities to the higher molarities, and the tarsi were rinsed after each few trials to avoid any collection of sucrose on the feet. After the five flies were tested in this manner, they were removed for feeding and watering, and a new batch of five water-sated flies was admitted to the chamber.

RESULTS

The flesh fly, *Sarcophaga bullata,* is easy to handle, because of its convenient size and relative docility. Flies mounted at 24–48 hours of age did not live as long on the mounting rods as those mounted at 96–120 hours. If the animals did not have an opportunity to get all the water they wanted before being anesthetized with ether, they died before or shortly after coming out of the anesthesia, as noted by Frings (1941). Flies that were water-sated before etherization were seemingly unaffected.

Temperatures a few degrees above 40° C. or below 15° C. affected the activity of the flies. The movements of the legs and the proboscis responses were slower than at temperatures of 37–39° C. and 19–21° C. The flies were most active at room temperature.

Approximately 2000 flies were used in this experiment, but over half of these were used to develop experimental techniques. Tests were made on 748 flies, 313 males and 435 females. These flies were subjected to 8241 tests at the three temperature levels.

The median thresholds, as determined by probit analysis (Dethier and Chadwick, 1950; Finney, 1952), were: at 19–21° C., 0.14 *M*; at 27–29° C., 0.06 *M*; at 37–39° C., 0.17 *M*. Significant differences between the per cent response of the flies at the various molarities were determined at the upper probability limits of 0.005 from the tables in Mainland (1948). The lower temperature compared with room temperature (27–29° C.) showed significant differences at 0.6 *M,* 0.4 *M,* 0.2 *M,* 0.1 *M,* 0.08 *M,* 0.06 *M,* 0.04 *M,* and 0.02 *M*. The higher temperature compared with the room temperature showed significant differences at 0.8 *M,* 0.6 *M,* 0.4 *M,* 0.2 *M,* 0.1 *M,* 0.08 *M,* 0.06 *M,* 0.04 *M,* and 0.02 *M* levels. No significant differences between the thresholds of the sexes were noted.

INTERPRETATIONS AND CONCLUSIONS

The median tarsal threshold for sucrose (0.06 *M*) for *Sarcophaga bullata* at 27–29° C. is relatively high when compared with that of some other Diptera. For instance, Hassett, Dethier and Fans (1950) reported a median threshold of 0.0098 *M* for the blow fly, *Phormia regina,* and Deonier and Richardson (1935) reported a median threshold of 0.025 *M* for the house fly, *Musca domestica.* Frings and O'Neal (1946), however, found the median sucrose threshold for *Tabanus* to be 0.06 *M* which is the same as that of the flesh fly. A possible explanation for the difference between the median thresholds of *Sarcophaga* and *Tabanus* and those of other Diptera might be that the food habits and habitats of these flies are different from those of the other species.

As to the question of absorption or adsorption in tarsal stimulation, no definite stand can be taken. From the results, it would seem that penetration was the factor involved at temperatures below room temperature, while at the higher temperatures adsorption was the factor. It is highly improbable, however, that different factors are involved at different temperatures. Perhaps the action, if any, of temperature on the peripheral threshold is masked by the action of temperature on central nervous functions, and thus on general behavior. Possibly neither adsorption *per se* nor penetration is involved, but instead discharge of surface potentials on the sensory hairs. This reaction would be basically independent of temperature. Such a phenomenon would show Hofmeister seriation of inorganic ions and seriations of organic compounds. In such a case, the effects of temperature would be entirely on other systems and thus would give no information of significance on the point in question.

SUMMARY

1. The effects of temperature on the sucrose thresholds of the tarsal chemoreceptors of the flesh fly were studied. Flies were mounted on rods and the thresholds determined in a constant temperature chamber at 19–21° C., 27–29° C. and 37–39° C.

2. A total of 748 flies (313 males and 435 females) were subjected to 8241 tests.

3. The median threshold at 19–21° C. was 0.14 *M*, at 27–29° C. was 0.06 *M*, and at 37–39° C. was 0.17 *M*.

4. On the basis of these data, no definite stand can be taken on the limiting mechanism of tarsal chemoreception.

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ALTERATIONS IN THE PROTEINS OF SEA URCHIN EGG HOMOGENATES TREATED WITH CALCIUM[1]

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The complex of processes involved in the occurrence of a precipitation reaction at the surface of exposed cytoplasm was named the "surface precipitation reaction" by Heilbrunn (1927). A survey of the properties of this reaction in a variety of forms is given by Costello (1932), and a related type of process in muscle fibers is described by Woodward (1948). The most recent treatment of the reaction (henceforth abbreviated SPR), including discussion of its physiological significance, is given in Heilbrunn's (1952) textbook.

The egg of the sea urchin *Arbacia punctulata* provides excellent material for the observation of the SPR. The egg is crushed in sea water, usually between a glass slide and a coverslip. Ordinarily, the egg breaks at one or more points, and the cytoplasm flows out at the site(s) of rupture. The outward movement of the exovates is halted by formation of a precipitation film, and this is usually accompanied by extensive vacuolization within the exovate mass. The Arbacia egg possesses numerous pigment-containing granules (really vacuoles), and these break down in the exovate. The granule lysis and vacuolization reactions are sometimes observed to travel wave-like across the entire cell.

The calcium ions of the sea water are responsible for the initiation of the SPR. If the calcium ions are removed by precipitation with oxalate or by complexing with citrate, the SPR does not occur, and instead, the cytoplasm of crushed eggs is rapidly and uniformly dispersed throughout the volume of medium available to it. This is true of the SPR in all forms studied, from the protozoan Stentor to the egg of the frog (Heilbrunn, 1952).

Heilbrunn (1952) has proposed a theory which attempts to explain such diverse phenomena as stimulation, narcosis, and cell division on the basis of changes in the state of the protoplasmic colloid. Thus, for example, cell division is always preceded by a gelation reaction, the so-called "mitotic gelation."

This physiological gelation process appears to be the analogue of the SPR described above. Indeed, Heilbrunn has proposed that the mitotic gelation is an "internal SPR," differing from the true SPR only in a lesser extent of reaction. Many of the events which accompany the viscosity increase of the cytoplasm in living cells do, in fact, have their striking counterparts in the events of the SPR (Heilbrunn, 1928).

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Hultin (1950a, 1950b), impressed by the possible important role of calcium in fertilization and in stimulation in general, studied the effects of the addition of Ca^{++} to homogenates of sea urchin eggs. The innovation of his method was the use of unfertilized eggs which had been freed, prior to their homogenization, of practically all of their ionic Ca. When Hultin added Ca in small quantities to such homogenates, several events were observed: a burst of oxygen uptake in great excess of uptake in the controls, the formation of acid, a sharp increase in the viscosity of the homogenate, and the breakdown of certain cytoplasmic inclusions. These events bear a suggestive resemblance to those which take place at fertilization (Runnström, 1951).

The experiment just described is, however, also a model of the SPR. Indeed, Hultin's preparation *is*, in a sense, the SPR, with the modifying condition that the rupture of the cells is temporally separated from the influx of calcium ions. Certainly, if the events enumerated above suggest those at fertilization, they duplicate the events of the SPR with even greater fidelity.

The experiments of Hultin therefore take on great interest in relation to all phenomena in which the "internal SPR" of cytoplasmic "gelation" are supposed to play a role. Now the SPR obviously involves the formation of an insoluble precipitation membrane (Heilbrunn, 1928), and is likely to be causally connected with a drop in the solubility of some cytoplasmic proteins. The formation of a fibrous gel, such as Hultin (1950b) and Gross (1952) describe would be difficult to account for in another way. Hultin was, however, unable to demonstrate any protein solubility changes as a result of addition of Ca.

It is obviously important to discover whether alterations in the solubility of cytoplasmic proteins do or do not in fact occur. Further, if such alterations can be demonstrated, then it is important that the reaction or reactions leading to this effect be analyzed at the molecular level. Such an analysis would serve to throw light upon the as yet obscure mechanism of the SPR and, perhaps, by extension, upon the processes of gelation and solation which are universal manifestations of vital activity at the cellular level.

This report describes experiments whose purposes relate to this analysis.

I wish to express my sincere gratitude to Dr. L. V. Heilbrunn for his assistance and encouragement during the course of this work.

The electron micrographs were made at the Electron Microscope Laboratory, Marine Biological Laboratory, by Mr. Delbert E. Philpott.

Materials and Methods

Eggs were obtained from female *Arbacia punctulata*. Samples from the pooled batches were tested for fertilizability and poor collections were discarded. The animals were usually injected in the oral region with 0.1–0.5 ml. of isotonic KCl. This treatment induced shedding of the ripe eggs but appeared not to affect immature females. The eggs were collected from six to ten females and quickly washed twice by centrifugation through filtered sea water in order to remove debris. A wash in sea water at pH 5.0 now served to loosen or dissolve the jelly coats, and these were carried away in the subsequent washings in Ca-free media. Washing by centrifugation and decantation five to eight times in Ca-Mg-free artificial sea water was followed by two washes in 0.35 *M* sodium citrate.

The eggs were now extremely fragile and were easily homogenized after centrifugal collection. Prior to this step, however, the eggs were cooled to 2° C. or below. The homogenization was carried out in a pre-cooled Potter-Elvejhem type of glass homogenizer. A few strokes of the tight-fitting pestle were sufficient. The process described resulted in homogenates of uniform consistency, with few if any intact cells.

As pointed out by Harris (1943), procedures of this type result in the rupture of a certain small percentage of the pigment vacuoles, although most of these bodies are resistant enough to withstand breakage in the course of homogenization. The homogenate was usually prepared with one part of packed eggs and one to ten parts of homogenization medium, this last being isotonic KCl, KCl with buffer (veronal-acetate-HCl), or Ca-Mg-free artificial sea water. The homogenates were strongly self-buffering at a pH of 6.5–6.9.

The details of the several experimental techniques are given in the appropriate sections of the experimental part of this report.

Experiments and Results

1. *Ionic specificity*

The first problem which arises in connection with the striking changes caused by the addition of Ca to the homogenates is that of ionic specificity. Since Hultin (1950b) was able to mimic several of the Ca effects by means of strongly hypertonic salt solutions, the possibility arises that the effects of Ca might be attributed simply to the increased ionic strength of its "isotonic" solutions as compared with that of isotonic solutions of uni-univalent salts. Thus, an isotonic solution of $CaCl_2$ (0.32 *M*) has an ionic strength of 0.96, given by the relation

$$\mu = 1/2 \sum_i c_i z_i^2$$

where μ is called the ionic strength, and the computation consists of summing the products, for each ionic species, of *c*, the concentration in moles, and z^2, the ionic charge [squared], and dividing the sum over all species by two. By comparison, an isotonic solution of KCl (0.48 *M*) has an ionic strength of 0.48. Since ionic strength, and not molarity, is the value of interest in kinetic salt effects, the above possibility must be investigated.

An aliquot of a homogenate was treated with Ca and a control aliquot was treated with a solution of KCl of identical ionic strength. Thus, 0.1 ml. of 0.32 *M* $CaCl_2$ was added to ten ml. of homogenate and 0.1 ml. of 0.96 *M* KCl was added to the control sample (10 ml.).

The control homogenate retained its normal appearance, whereas the material in the experimental tube underwent, within fifteen minutes, a marked precipitation reaction, as a result of which the viscosity of the homogenate (as a whole) rose. The Ca-treated homogenate formed fiber-like aggregates which adhered to the sides of the test-tube. The granule lysis reaction was evident, and the homogenate underwent a color change from orange-red to a deep wine-red. As will be shown below, the color change could be prevented by strong additional buffering of the homogenate at pH 7.0.

2. *Solubility changes*

Hultin (1950b) was unable to observe any alterations in the protein solubility of his homogenates as a result of Ca-treatment. He assumed that such changes do occur, but that the eventual loss of protein from the soluble fraction of the homogenate was balanced and even over-compensated by the release of trichloroacetic acid-insoluble N from the cytoplasmic inclusions which lyse in the presence of Ca.

There is, however, a simple method by which the difficulties caused by granule lysis can be overcome without removing the granules during the reaction period (this latter step constituting a partial fractionation of the homogenate which is not desired at this stage of the investigation). Harris (1943) showed that the pigment granules of the sea urchin egg behave osmotically. Now it is these granules, or vacuoles, which contribute the major quantity of trichloroacetic acid- (henceforth "TCA") insoluble N to the homogenate upon their lysis. Thus, if the granules could be osmotically ruptured in *controls as well* as in Ca-treated homogenates, the phenomenon of granule lysis should have its effect cancelled, and solubility changes in the initially soluble cytoplasmic proteins alone should be measurable.

It is possible to produce osmotic lysis of over 85% of the pigment granules by the addition of water to the homogenate *after* the treatment under investigation. In this way, the effects of Ca^{++} upon the soluble proteins of the homogenate can be studied.

The same basic experimental design was used in all of the solubility experiments. Homogenates were treated with the agent suspected of effecting a solubility change in the proteins. Controls were treated with uni-univalent salt solutions with ionic strengths equal to those obtaining in the experimentals. The tubes containing the homogenates were incubated, either at 2° C. or at 21° C., for various periods. At the end of the incubation period, the homogenates were treated with twice their volume of ice-cold distilled water. This brought about the desired lysis of remaining pigment granules. The homogenates were now diluted with (usually) ten volumes of ice-cold distilled water or *M* KCl, buffered in some cases to pH 7.0. Extraction was carried out in the icebox at 1° C. for one to twenty-four hours. At these low temperatures and relatively great dilutions, the aggregation reactions initiated by Ca were brought to a practical halt.

At the end of the extraction period, the tubes were spun in a Servall anglehead centrifuge at 17,000 to 20,000 g. The clear supernatants were analyzed for their total protein content by one of several methods. The experiments were thus set up to reveal differences in solubility or, more precisely, in sedimentability, the latter being a function of the relative states of aggregation of the soluble or suspended proteins.

For the comparative results required, a quantitative modification of the biuret test, slightly modified from Fine (1935), was found adequate. The results were reproducible, and the slight turbidity which tends to develop at the higher protein concentrations can often be avoided by suitable dilution and temperature control. Only measurements obtained upon visually non-turbid systems were admitted.

Most of the experiments were repeated with analysis by spectrophotometry. Aside from offering a means of estimating total protein, the absorption spectra of the water extracts provide information concerning the relative distributions of protein and nucleic acid in experimentals and controls.

When the experiment described was performed, there was always a measurable precipitation of protein or protein containing material from the homogenates which had been treated with Ca. At least, at every given relative centrifugal force, more protein was sedimented in the experimentals than was in the controls. When the homogenates were relatively concentrated, that is, when the dilution with homogenization medium was less than five times, the reaction was very rapid at 21° C., although not "instantaneous" in the way that ionic precipitation reactions often are at room temperature.

Depending upon the conditions obtaining, from zero to ninety per cent of the total protein extractable from the whole homogenates by water could be precipitated. Actually, the extraction medium is a very dilute salt solution, since the homogenate itself contains considerable salt.

For reasonable reaction velocities at 21° C., concentrations of Ca^{++} in the range of 0.001 to 0.01 were required. The significance of these levels will be discussed below.

The results suggest that the rate, but not the final extent of solubility loss is dependent upon the concentration of Ca; but this effect could not be noted if dilutions were too great, since the over-all velocity of precipitation is very sensitive to dilution of the homogenate. Indeed, homogenates made with one part of eggs to more than ten parts of medium often failed to give a measureable rate of precipitation in the presence of 0.01 M $CaCl_2$, although other Ca effects, such as granule lysis, were observed.

In many experiments where the reaction was permitted to proceed only a short time after treatment with Ca^{++} and a wide range of Ca concentrations was added, the following phenomenon was observed. Plots of solubility loss as a function of Ca^{++} added were often not simple, concave-downward curves, but rather, showed a maximum at about 0.5–1.0 millimols/liter, followed by a local minimum and finally by steadily increasing values asymptotic with approximately 90% precipitation. The amount of protein lost from the homogenate at the low concentration maxima, when these occurred, was of the order of 20–30% of the total protein extractable with water from the controls.

This is demonstrated by the data in Table I, which shows the results of two typical experiments carried out in successive summers at Woods Hole. Both homogenate preparations were made from 1 : 6 homogenates, *i.e.*, one part of packed eggs homogenized in five parts of isotonic KCl. The considerable variation from one homogenate preparation to another is illustrated by the two sets of data, as are the general characteristics of the relation between solubility loss (or increased sedimentability) and the concentration of added Ca^{++}.

Spectrophotometric methods corroborated the results of the chemical determinations. The water extracts of experimental and control homogenates were centrifuged at 17,000 g for 10 minutes to one hour in the cold, thus brought to water-clarity, and then diluted and read directly in the Beckman spectrophotometer.

TABLE I

Loss in the water solubility of homogenate proteins in the presence of varying amounts of calcium ions. Two separate experiments

Experiment number	Concentration of Ca in mM/liter	Per cent loss in solubility
1A	0.00	0.00
1B	0.57	38.8
1C	5.70	33.4
1D	57.0	72.2
1E	113	81.5
1F	170	94.4
2A	0.00	0.00
2B	1.00	19.0
2C	5.00	14.8
2D	10.0	37.0
2E	100	91.6
2F	500	90.5

Complete absorption spectra in the wave-length region 220 to 300 millimicrons were made for the experimentals and for the controls. The optical density at wave-length 280 millimicrons is a measure of the protein (and aromatic amino acid) content of the sample. The effects of the presence of nucleic acid and of scattering do not introduce a serious error in comparative measurements such as were made here.

In every case, a loss in protein from the Ca-treated homogenate occurred, and was indicated by a reduced optical density at 280 mμ. The absorption spectra had interesting features aside from the changes in density at 280. Figure 1 illustrates the result of a typical experiment. Here, a 1 : 5 homogenate was treated with 0.03 *M* $CaCl_2$ and the control was treated with a solution of KCl of equal ionic strength. After five minutes of incubation at 21° C., each tube was diluted with five volumes of ice-cold distilled water. The extraction was continued in the ice-box for four hours. At the end of this period, both tubes were centrifuged at 17,000 g for ten minutes. The clear supernatants were removed and further diluted with water buffered to pH 8.5 with bicarbonate. Absorption spectra were made in the ultraviolet region.

The upper curve (half circles) in the figure represents the absorption spectrum of the control extract, while the lower curve (solid circles) is that of the experimental. In both cases, the spectrum is that of nucleic acid plus protein, with a peak at 260 millimicrons and an appreciable absorption at 280 millimicrons. Obviously, an over-all loss of water-soluble material has occurred in the experimental system. In addition, the shapes of the curves suggest that the composition of the precipitated material differs slightly from that of the supernatant as a whole, as represented by the upper curve.

The ratio of the molar extinction at 280 mμ to that at 260 mμ has a characteristic magnitude for most proteins and a different, but equally characteristic, magnitude for nucleic acids. Thus, for PNA, this value is in the neighborhood of 0.5, whereas for protein alone it is more likely to have a value near 1.75.[3]

[3] This is not the case for proteins of unusual amino acid composition, such as, for example, protamine.

Warburg and Christian (1942) used these facts as the basis of a method of estimating the relative quantities of protein and nucleic acid in a mixture of the two.

Optical density, the quantity measured in these experiments, is given by the relation

$$D = kcd$$

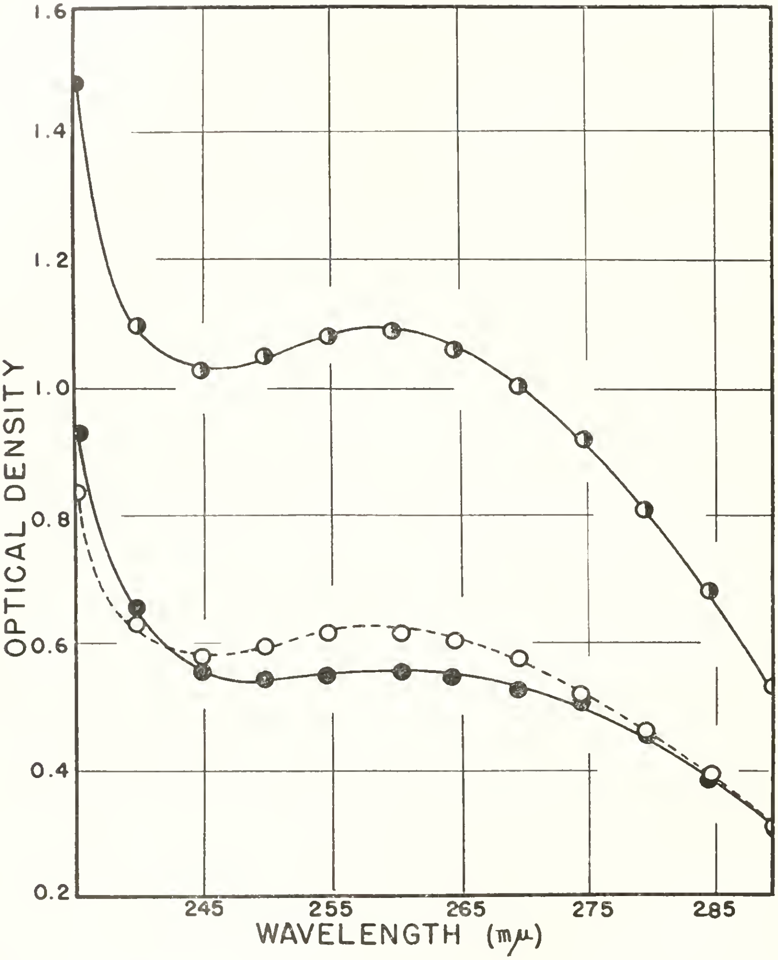

Figure 1. Ultraviolet absorption spectra of water extracts of sea urchin egg homogenates. Half circles: control. Full circles: experimental, treated with Ca^{++}. Open circles: calculated curve for experimental. See text.

where *D* is the optical density, *k* is a specific extinction coefficient, *c* is the concentration of absorbing substance, and *d* is the length of the light path through the sample. The extinction ratio mentioned above is readily obtained from the measured "*D*" values. For a single sample, the optical density will differ at the two wave-lengths, but the concentration of chromophore and the light path do not change and are therefore constants, so that

$$\frac{D_{280}}{D_{260}} = \frac{k_{280}}{k_{260}},$$

which is the desired ratio.

The value of this ratio is 0.74 for the upper (control) curve and 0.83 for the experimental curve in Figure 1. This is a significant difference, and indicates that the lower curve represents a chromophore which is relatively poorer in nucleic acid than is the control. But the spectra have been recorded for water extracts, and thus the insoluble, aggregated material in the experimental homogenate must be *richer* in nucleic acid than that in the control.

It is possible, by preparations of several dilutions of the control, to obtain mean values of k (independent of concentration) at the several wave-lengths. These values are multiplied by $d (= 0.996$ cm.) and by "c," which is now that fraction of the absorption (at 280 millimicrons) of the control shown by the experimental. This is equivalent to assuming that the differences between the control and the experimental absorption spectra can be accounted for on the basis of the changes indicated by the 280 mμ absorptions. The product $(k \cdot c \cdot d)$ gives a series of theoretical values for D, and these values are plotted for purposes of comparison with the experimental absorption spectrum. Such a calculated curve is shown by the open circles in Figure 1.

Although, due to the large absorption by nucleic acids even at 280 millimicrons, this type of manipulation can give no quantitative data on the distribution of nucleic acid and protein in the system, it does qualitatively indicate that the experimental water extract is relatively poorer in a component absorbing strongly at 260 mμ than the control extract.

Finally, a preliminary localization of the reaction was attempted in the following way:

A homogenate was centrifuged in the cold at 2000 g for 20 minutes. This deposited a layer of red granules, and no red pigment was left in the supernatant fraction. Treatment of these supernatants was the same as that for whole homogenates.

In every case, the addition of calcium to this supernatant layer brings about a solubility loss on the part of protein components. Figure 2 gives the spectrophotometric analysis of one such experiment, "c" represents the water extract of the control, and "x" that of the Ca-treated supernatant.

Plainly, the material which becomes insoluble in the whole homogenate comes, at least in part, from the fraction devoid of the largest granules.

3. *Acid formation*

The appearance of acid upon fertilization of the egg of *Paracentrotus lividus* was first reported by Runnström (1933), and this was confirmed for *Psammechinus miliaris* by Borei (1933) and by Laser and Rothschild (1939). These observations led Hultin (1950b) to test for the appearance of acid upon the addition of Ca to his homogenates. Hultin was successful in demonstrating this effect.

The acid formation has considerable significance for the SPR, and accordingly an attempt was made to measure it in our own material.

A homogenate was prepared in the usual way, and then frozen in dry-ice and acetone and dried *in vacuo*. Later, this material was reconstituted with ice-cold distilled water. This material was self-buffered at pH 6.55, as measured with the glass electrode. When the homogenate was brought to 21° C. and treated with $CaCl_2$ (final concentration = 0.005 *M*) the material changed color from brown to deep red within a few minutes. The pH of the system fell from 6.55 to 5.50 within 90 seconds. It is easily calculated that, even with buffering neglected, this large release of H^+ cannot be accounted for on the basis of the slightly acid pH of the added $CaCl_2$.

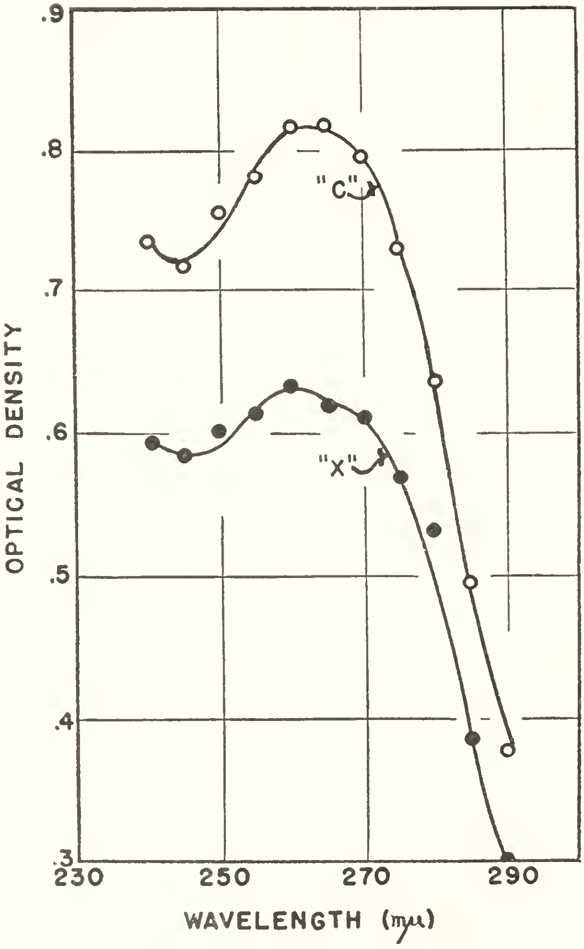

FIGURE 2. Ultraviolet absorption spectra of water extracts of homogenates free of pigment granules. "c" = control. "x" = Ca^{++} — treated experimental.

The data indicate that even in the presence of the considerable buffer capacity of the homogenate proteins, etc., the hydrogen ion concentration increased more than ten times within the first 1.5 minutes after the addition of Ca. The acid formation must thus be of considerable magnitude. In the light of these observations, it is important to ask whether the change in pH is not the factor responsible for the precipitation of protein by Ca.

That this is not the case is easily demonstrated in experiments in which the homogenates are strongly buffered at pH 7. In these systems, the color change does not take place and, of course, the pH remains close to 7, but the loss of protein solubility under the influence of Ca is unaffected.

The matter of color change is of interest, because in the SPR as observed in crushed eggs, the breakdown of the pigment granules is accompanied by a pronounced reddening of the protoplasm of the exovate and often of the whole cell, as the wave of granule lysis sweeps across it. This change in the pigment (echinochrome) released from the vacuoles is particularly striking when eggs are crushed in media somewhat richer in Ca than is sea water. This effect is undoubtedly due to the change in pH of the regions of the cell effected by the SPR.

Two homogenates were prepared and treated in the usual way, except that one was strongly buffered at pH 7.15 with tris-(hydroxymethyl)-aminomethane while the other was unbuffered except by its own ampholytes. Both were treated with the same quantity of Ca^{++} (0.005 M) and incubated for a few minutes. The precipitation reaction took place to approximately the same extent in each. Both tubes were centrifuged to sediment the solids, leaving faintly turbid supernatants. These were diluted somewhat and their pH values measured once again with the glass electrode. Absorption spectra of the two preparations were made in the visible region of the spectrum.

The results of the spectrophotometric measurements are plotted in Figure 3. Clearly, the system whose pH was 6.5 was red, while the one with pH = 7.15 was yellow-orange. This shift in absorption is, as closely as it is possible to judge, the same type as that which occurs when the SPR is observed in cells. These color changes are, of course, due to the presence of the pigment echinochrome, which is a pH indicator, apparently even when in combination with its protein ligand in the homogenate or in the egg.

4. *Release of non-protein nitrogen*

Woodward (1949) reported experiments in which various fractions of Arbacia egg granules were assayed for proteolytic activity. He found low but significant proteolytic activity, increasing from 35 to 50 per cent upon addition of cyanide, in the "granular" fraction of the eggs. Ca-activation of the protease(s) was not demonstrated.

Gross (1952) reported in a note that the whole homogenate of Arbacia eggs contained a Ca-activated protease, with a pH optimum near 6.6. Lundblad (1952), in the course of his extensive investigations upon the proteolytic activity of sea urchin gametes, reported that the weak proteolytic activity of extracts and homogenates of unfertilized eggs was increased by Ca.

Because of the possibility of a relation between proteolysis and coagulation, and perhaps precipitation, of proteins in the homogenates, it seemed worthwhile to attempt a further step in the analysis. Lundblad's assays of protease activity were made by a viscosimetric method. In the light of many investigations such as those of Örström (1941), demonstrating changes in the non-protein nitrogen (NPN) of eggs after fertilization, it seemed further indicated that one should determine whether NPN increased directly after the addition of Ca to the homogenates. The method used by Lorand (1952) in his studies on fibrinogen was adapted for use in the homogenate system.

Homogenates were prepared in the usual way. Calcium was added in amounts sufficient to give a reasonable rate of precipitation. At intervals, one of the several tubes employed in the experiment was treated with two to five volumes of cold distilled water. An equal volume of 2% monochloroacetic acid was added. After several minutes of gentle shaking, the proteins were precipitated with trichloroacetic acid in a final concentration of 5%. The precipitation was permitted to proceed for 24 hours in the icebox. Finally, the flocculent precipitate was sharply centrifuged down and the clear supernatants collected. The NPN of these super-

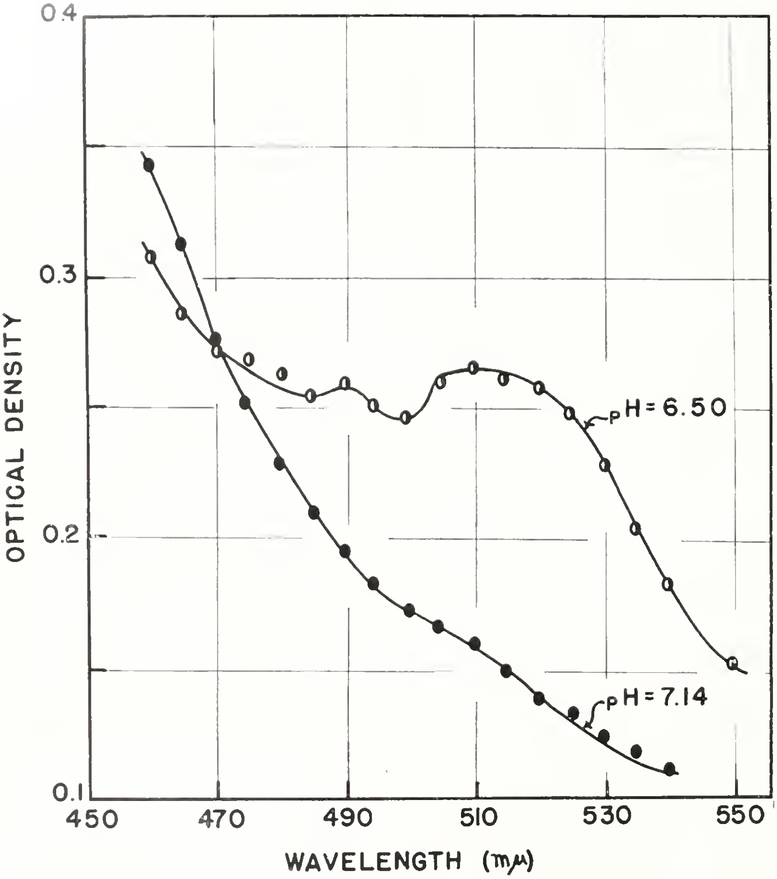

FIGURE 3. Visible light absorption spectra of Ca^{++} — treated homogenate extracts. Half circles: unbuffered. pH = 6.50. Full circles: buffered at pH 7.14.

natants was determined after digestion in boiling H_2SO_4 and "Superoxol" by direct nesslerization.

Small and very variable increases in NPN were observed to follow the addition of Ca to the homogenates. When measurements were made within the first hour after Ca treatment, the increments were usually of the order of 2–3 per cent, or barely within the limits of precision of the method. With increasing time, however, more NPN appeared. The controls showed little or no protease activity in the absence of Ca when NPN release was the criterion of activity. When the experiments were continued for several hours, however, the controls also showed increments in NPN at the end of the experiment (as compared with a primary control determination at zero time). These increments were always smaller than those observed in the homogenates which contained Ca.

Thus, for example, in an experiment which ran for six hours, the experimental homogenate, which was treated with 0.01 *M* $CaCl_2$, contained 17.3 mg.% NPN (the value for the final diluted assay sample). The control, run along with the experimental, contained 15.6 mg.%, while the primary control, which had been precipitated with TCA six hours previously, contained 14.6 mg.%. These values are the averages of triplicate determinations.

A typical result for an incubation period of 24 hours (at 1° C.) is the following:

Material: Ca-free homogenate, lyophilized and reconstituted.
Treatment: Experimental—0.02 *M* Ca^{++}
Control—KCl of ionic strength equal to that of added Ca solution.
Result: Control: 31.0 micrograms of NPN/ml.
Experimental: 52.2 micrograms of NPN/ml.

5. *Electrophoretic experiments*

Although electrophoretic examinations of sea urchin egg proteins and protein fractions have been reported previously (Monroy, 1950; Monroy and Monroy Oddo, 1951), earlier work dealt with frozen and dried material. Since the publication of the critical experiments of Lindvall and Carsjö (1951), it has become important to re-examine these results in the light of possible artifacts produced by the lyophilization of fertilized and unfertilized eggs which retain their internal as well as external calcium.

Electrophoresis of extracts of homogenates such as are used in the present work promises to be a useful tool in the further analysis of the SPR-type reactions. The method employed in the experiments was as follows:

Two aliquots of homogenate were obtained. One was calcified, and the other, the control, was treated in the usual way with KCl. After a few minutes incubation, there was the usual treatment with distilled water, followed by extraction in the cold with distilled water. The extracts were finally centrifuged for twenty minutes at 17,000 to 20,000 g. Only clear or very faintly turbid supernatants were accepted for electrophoretic analysis. These were placed in 1-cm. diameter dialysis tubing and dialyzed in the icebox (1° C.) for 48 hours against several changes of 500 ml. of 0.1 N $NaHCO_3$ at pH 8.5 (21° C.). The thoroughly dialyzed colloids were then submitted to electrophoresis in the Perkin-Elmer Tiselius-type instrument, using the 2-ml. cell. The cylindrical lens system was used for the photographic records of the runs. Photographs were made at definite intervals with the current temporarily turned off. For analysis of these data, the negatives were placed in a photographic enlarger and projected, and the enlarged patterns were traced on cross-section paper. The pattern shown in Figure 4 was prepared in this way, and then inked for reproduction.

The choice of bicarbonate buffer calls, perhaps, for justification. This system was used by Monroy and Monroy Oddo (1951) in their recent experiments upon the protein fractions of fertilized and unfertilized sea urchin material. Their patterns seemed in general to show satisfactory separation. To facilitate comparison, bicarbonate was used in the present experiments. The separation (resolution) was not as good as could be desired, although sufficient for the comparative data needed. Migration was quite rapid.

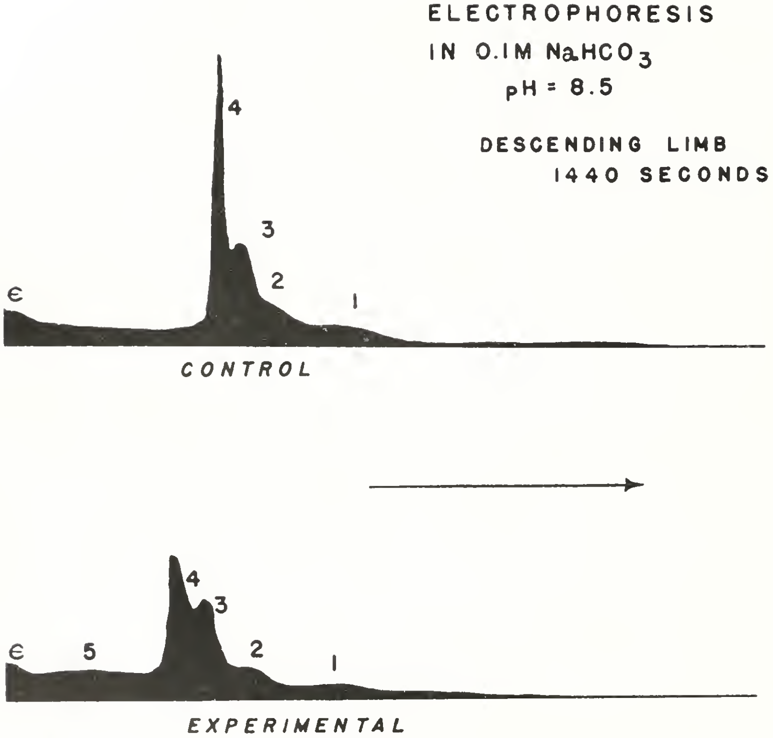

FIGURE 4. Electrophoresis of water extracts of homogenates. Control, untreated. Experimenal, treatment with Ca^{++}.

Figure 4 shows a typical result. The pattern marked "control" is that of the water extract of a control homogenate. The "experimental" pattern was given by the water extract of a Ca-treated homogenate. Both patterns were photographed at the same interval relative to the commencement of the run. The descending limbs are shown. The field strength (potential gradient) was 6.5 volts/cm. It is apparent that the migration was rapid, and that acceptable resolution was achieved after only 1440 seconds.

In general, three boundaries were observed in all experiments (marked "2," "3," and "4"), with a suggestion of a fourth ("1") in some. Better resolution of these boundaries has already been achieved in experiments with buffers at a lower pH (to be reported elsewhere). In two experiments, a suggestion of a very low, rapidly spreading boundary (marked "5" in Fig. 4) was given by the experimental extracts. In all of the experiments, all boundaries other than the slowest and largest (marked "4") seemed unaffected by the precipitation reaction which had taken place in the experimental. The loss in area of boundary "4," as illustrated in the experiment described, may possibly be ascribed to a selective interaction of the component represented by the boundary with calcium. It remains possible that losses occurred in the smaller boundaries and are not detectable. Nevertheless, the major effect of the calcium ions seems to be upon the largest component.

6. *Electron microscopy*

If the precipitation reaction under investigation does, indeed, involve one or more specific fractions of the cell's macromolecular or particulate population, then this should be demonstrable by direct visualization of the insoluble products of the reaction. The electron microscope makes such visualization theoretically possible.

Dilute homogenates were prepared. Ca was added to the experimentals, and the controls were treated with KCl of equal ionic strength. Small drops from the experimental and control tubes were immediately withdrawn and placed on collodion films deposited on copper grids.

When the "SPR" in the experimentals had proceeded for the proper interval of time, the reactions were stopped by flooding the mounts with distilled water and removing the large drops formed with filter paper. This process was repeated several times, with increasing intervals between flooding and withdrawal of the distilled water. This process served to 1) dissolve away the salts, 2) lyse the large pigment granules and wash away their ghosts, and 3) remove all water-soluble parts of the preparation. In addition, the mechanical disturbance occasioned by the withdrawal of the drops removed all but the most tenaciously sticky materials from the collodion.

The samples were either permitted to dry immediately, or they were first fixed with 0.2% phosphotungstic acid for two minutes and then washed again and dried. Finally, all preparations were shadow-cast with chromium. The electron micrographs were made with an RCA model EMU microscope.

Fixed and unfixed preparations gave essentially the same results, except that the materials fixed with phosphotungstic acid showed particles which were slightly more discrete and less flattened. Some slight syneresis was observed in these preparations. The discreteness and lesser degree of flattening are probably due to a certain rigidity imparted to the proteins by the fixative action of the acid, rather than by its electron stain properties. The photographs presented in this report were all made from fixed preparations.

Figure 5 is typical of the results obtained with the control preparations. Whether fixed or unfixed, these preparations showed only a thin layer of material blanketing the collodion substrate. The washing procedure appears, in these preparations, to have removed not only the water-soluble materials, but in addition

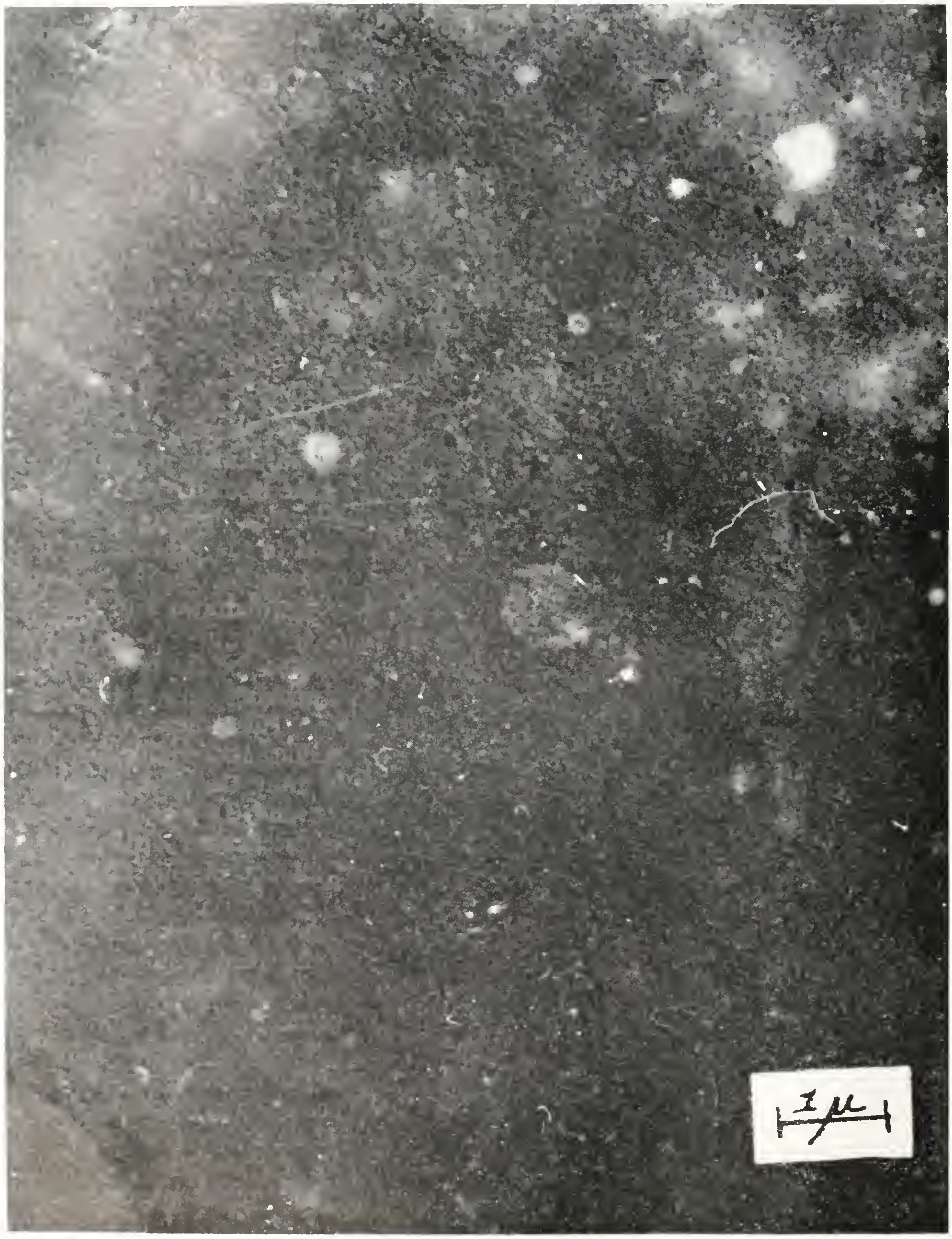

FIGURE 5. Electron micrograph of control preparation. See text. Magnification: 15,000 ×. Collodion surface with some low-molecular materials.

most of the water-insoluble materials. This is a result of the mechanical disturbance effected by the repeated withdrawal of the water droplets. Such a lack of larger structures in the control preparations is indeed fortunate, for such materials as do appear as a rule in the experimental preparations and are absent from the controls must in some way have had their solubility in water or their adherence to collodion sharply altered by the treatment with Ca. The specimen in Figure 5 is essentially clean collodion with perhaps some deposit of relatively low-molecular material. The magnification is 15,000 diameters.

Figure 6 is a typical experimental specimen, seen at a magnification of 28,000 diameters. Plainly, the result of the Ca-treatment has been the formation of a flocculent precipitate, insoluble in water, and involving particles other than the large pigment granules, which have been lysed and washed away by the preparation method. At the magnification shown, it is not possible to resolve the fine-structure of the microflocs to any appreciable extent; however, the edges of the floc shown, as well as the very small aggregates on the collodion, suggest that the basic aggregating unit is small and approximately spherical. Although there is a suggestion of linear aggregation on the figure, there is in general no pronounced order in the flocs as observed. They appear always, at this magnification, to be of the close-packed, polyfunctional type.

When observed at the highest magnifications, such as that in Figure 7 (110,000 ×), the thinnest microflocs reveal their fine-structure. The "linear" aggregates seen only poorly at the lower magnifications dominate these pictures as long, ropy strands of material, made up of (highly flattened) spheres in linear array. Since high surface pressures are exerted upon the specimens with the air-drying technique used in these preparations, it is likely that the discs were originally spheres. The aggregation reaction may thus consist in part of a difunctional type of polymerization at the earlier stages and then an irregular and extensive interchain packing superimposed.

It is probable that the extreme irregularity of the final packing in the microflocs observed is a result of 1) the drastic treatment of the material during the preparation of the specimens, and 2) syneresis due to the fixative. Of course, since the particles in question do not stick (probably because they are not aggregated) in the control, the foregoing considerations do not apply to controls.

Several representative plates at the high magnification (electronic magnification = 13,250 ×) were subjected to microscopic examination and measurement of the images of the small particles making up the aggregates. These particles, which it should be remembered do not remain on the control mounts, are distributed in diameter about a mean value of 380 Å. The distribution is skewed with the mode displaced somewhat toward the smaller diameters, and the variance is rather large (S.D. = 80 Å.).

The large variance, although not unusual in electron microscopic preparations of cell fractions, indicates, probably, rather extensive deformation of the original particles.

Discussion

The objectives of these investigations were two-fold: first, the clarification of the question of protein solubility changes as a result of the "test-tube SPR" and

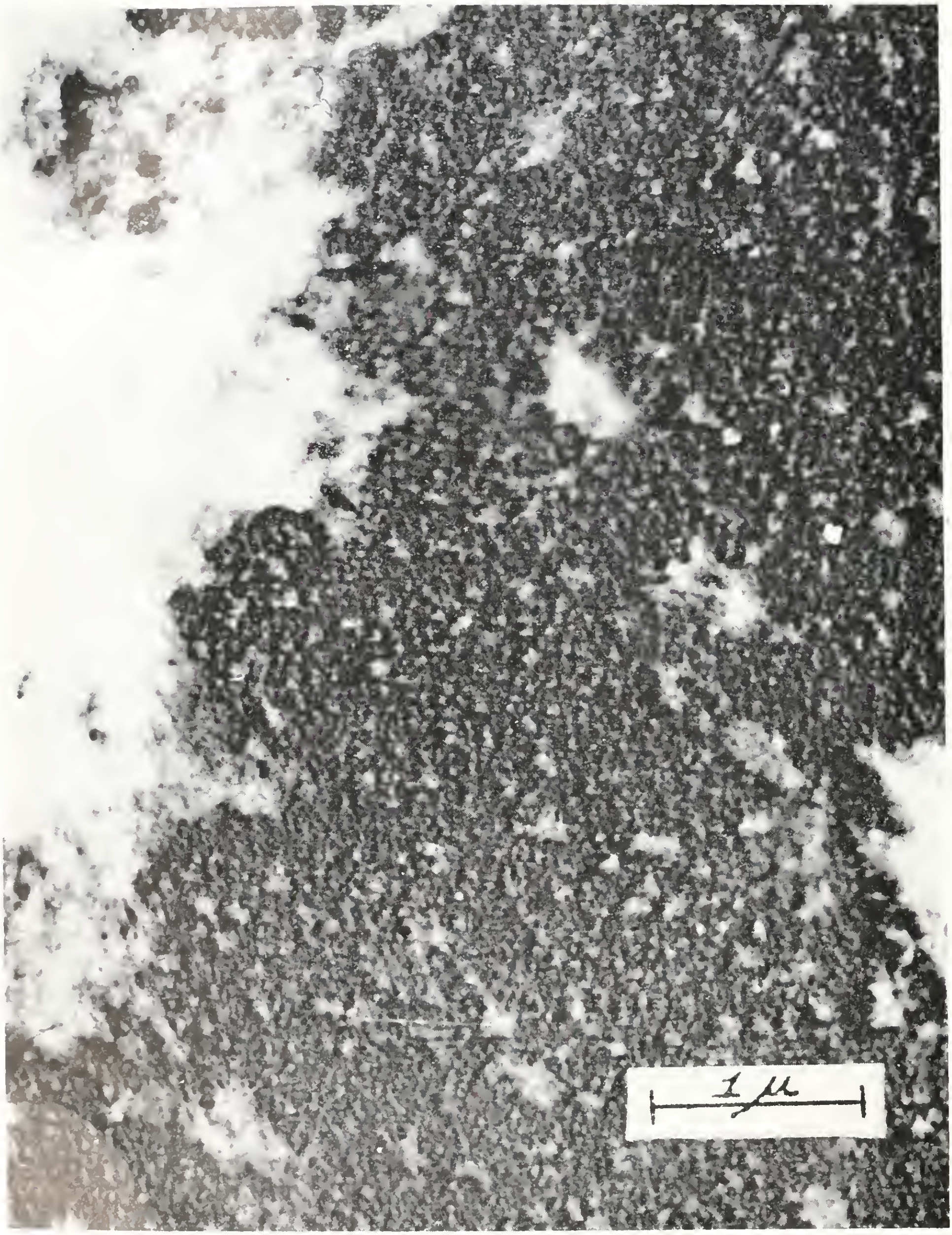

FIGURE 6. Electron micrograph of experimental preparation. See text. Magnification: 28,000 ×.

second, the construction of a reaction mechanism for the over-all phenomenon. By extension, such a mechanism would be of great utility in interpreting the molecular basis of colloidal alterations in the cytoplasm of living cells.

The addition of calcium ions *has* been found to reduce the solubility of some proteinaceous constituent of the homogenate, or at least, the sedimentability of this constituent is increased, which can be interpreted as an increase in aggregation.

But the second objective remains remote. One has had, so far, to be content with the acquisition of a spectrum of *possible* reactions involved in the macroscopic event called the SPR. Thus the experiments reported make no attempt to test interrelations of reactions, but rather to establish that certain reactions do occur as a result of the initiation of a "test-tube SPR" by Ca.

The fact that a local level or optimum for precipitation exists in the homogenate with regard to concentrations of Ca added at the level of 0.001 to 0.01 *M* may be related to the following facts.

1) The "physiological" rates of reaction (with half-time of the order of seconds to minutes) at 21° C. are the ones observed at these concentration levels.

2) The free Ca of unfertilized eggs is of the order of 0.0005 *M* (Mazia, 1937). Also, the concentration below which an SPR (in whole eggs) will *not* take place is very close to this, *viz.,* 0.0003 *M*. When the *free* Ca concentration of the cytoplasm is increased beyond this general level (5×10^{-4} *M*), the sea urchin egg experiences a protoplasmic gelation, or clotting. Mazia (1937) demonstrated that the fertilization of sea urchin eggs results in the liberation of *free* (ultrafilterable) calcium in the egg. Furthermore, the concentration of free Ca in the egg was shown to increase by 0.001 *M* upon fertilization, placing the total free Ca of the fertilized egg at 0.0015 *M*.

It may be significant that this level of Ca concentration, which we may term "physiological" for the system studied, is at one end of the range indicated as required for reasonably rapid rates of reaction in the *in vitro* system. Considering the dilution of reactants which takes place in the preparation of an homogenate, the correspondence of these concentration levels is surprisingly good.

The fact that the precipitated material contains nucleic acid may explain why such large proportions of the total cellular protein can ultimately (at very high Ca concentrations) be aggregated, and still appear to comprise, through most of the concentration range, a single fraction or group of fractions closely related. First, it is safe to assume that most of the nucleic acid being precipitated is PNA. Villee *et al.* (1949) have shown that the DNA of the uncleaved Arbacia egg corresponds to only about 6% of the total nucleic acid, and according to Abrams (1951) this figure is probably too high. The small quantity of DNA present in the homogenate could then hardly account for the large proportion of the total nucleic acid which can be precipitated by small quantities of Ca. Finally, if the affected fraction contains PNA, it may be one of the PNA-rich particle fractions of the cytoplasm, in which case it could conceivably represent a large proportion of the total protein of the egg.

The electrophoretic experiments suggest that the fraction(s) undergoing change under the influence of Ca are a part of one of the largest macromolecular or small-particulate fractions of the cell. The electron microscope pictures show that the particle in question is (after reaction with Ca) a large macromolecule or a very small complex cytoplasmic particle and that it is probably globular before polymerization.

It is specified that the particle has these characteristics *after* treatment with Ca, because a careful inspection of high magnification photographs, such as that in Figure 7, reveals a fine-structure within the particles themselves, at the level of 40–70 A. Probably, this represents the fine structure of the particles, but there is no evidence that the 380 Å particle is not *itself* an aggregation product resulting from the addition of Ca. The latter is, however, rather unlikely for many reasons, among which the results of the electrophoretic experiments and the probable large decrease in entropy in such a specific aggregation are prominent.

Thus, the experimental evidence seems to indicate a process in which Ca brings about the aggregation of certain specific particles of the cytoplasm into an insoluble coagulum. The specificity of this reaction, as well as the quality of its insoluble product, fit well with what is known of the SPR and of its normal analogue, the cytoplasmic sol-gel transformation.

The other events of the test-tube SPR also have their analogues in the SPR of the whole cell, and, interestingly, in the events of fertilization and stimulation. Whether the acid formation observed in the system under investigation here is related to that which occurs at fertilization (Runnström, 1933) is, of course, not decided by these experiments. Nevertheless, there is a strong suggestion that this might be the case. The color changes appearing in the test-tube reaction have their direct parallel in the SPR, and suggest that injury can produce large local increases in the hydrogen ion concentration of protoplasm. This is, of course, a fact generally recognized from other types of experiments (*e.g.*, Chambers and Pollock, 1927). The proteolysis measured in the *in vitro* system has its analogue in fertilization (Lundblad, 1950) and, *in vivo*, probably in the form of a release of NPN (Örström, 1941).

It is perhaps significant for the evaluation of the "colloid chemical" theory of stimulation of Heilbrunn (1952) that the *in vitro* system here studied has similarities to the clotting of blood in two heretofore undemonstrated areas. Not only do both reactions involve initiation or activation by Ca ions, but both involve polyfunctional polymerizations of macromolecular monmers, and both involve some step in which a proteolytic enzyme is activated and small quantities of NPN are released. This must not be taken to indicate that the precipitation reaction in the homogenate is necessarily mediated by a proteolytic enzyme. Such an enzyme is indeed present, but its participation in the reaction leading to aggregation in the homogenate must be demonstrated by methods not reported here. Such methods, particularly the use of alternating low and high temperatures, and the addition of clotting inhibitors are currently in use in this laboratory. It is also interesting, in this connection, that the fat solvent anesthetics such as ether and alcohol inhibit the *in vitro* reactions between the homogenates and calcium. These results will be more fully reported in another publication.

It should be noted that the formation of true fibers, such as those of fibrin (Hawn and Porter, 1947), is not observed in the SPR-type reaction. Plainly, the fibrin clot type of structure could not, and does not occur within a living cell. It is for this reason that it is erroneous to speak of cytoplasmic gelation or of "protoplasmic clotting." The word "gel" defines a system of infinite viscosity,

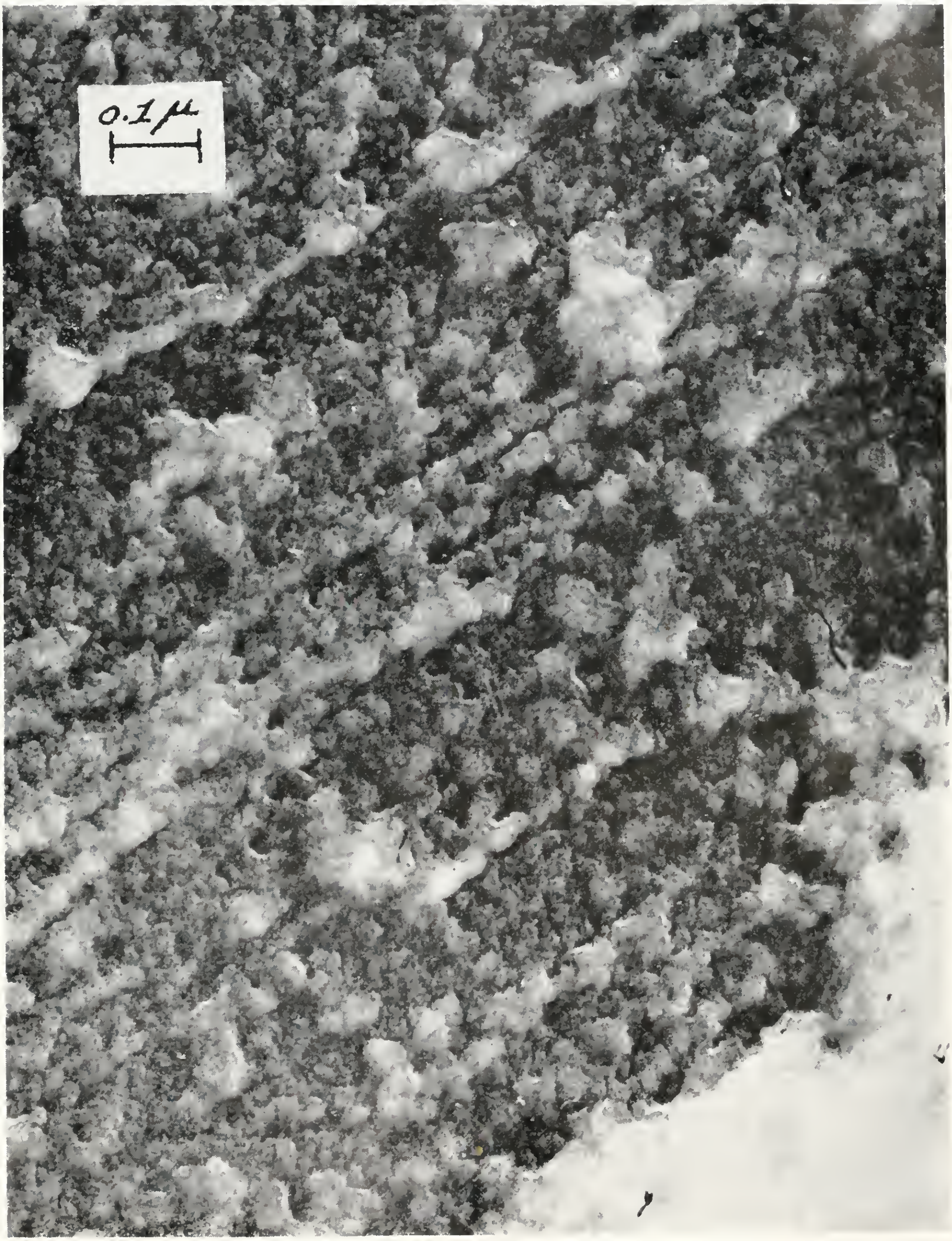

FIGURE 7. Electron micrograph of experimental preparation. See text. Magnification: 110,000 ×.

which is the case with a normal fibrin clot. But in a living cell, the cytoplasmic viscosity increases only by a factor of three to six in the usual sol-gel transformation. One presumes that in the SPR, these changes would increase in magnitude, and could indeed lead to a gel.

From these considerations, one may conclude that an aggregation process that might be expected to occur in a living cell, or even in the SPR, should certainly not lead to the formation of fibers of great length. Rather, the expectation is a reversible aggregation of small units into networks of dimensions easily accommodated within the confines of a single cell. The nature of the aggregation process observed in the homogenate system is thus, at least teleologically, justified.

SUMMARY

1. The addition of Ca^{++} to homogenates of Arbacia eggs which have been previously made calcium-free results in the loss in solubility of certain protein constituents of the homogenate.

2. The precipitated material is rich in nucleic acid, and comes, at least in part, from the fraction non-sedimentable at 2000 g.

3. The addition of Ca^{++} to homogenates activates a proteolytic enzyme and leads to the release of small amounts of additional non-protein nitrogen.

4. None of these reactions can be ascribed to the high ionic strength of solutions of calcium salts as compared with that of isosmotic solutions of uni-univalent salts.

5. The fraction undergoing solubility loss appears to be electrophoretically homogeneous, since it is represented by the largest and slowest-migrating boundary in the water extract of the homogenate.

6. Electron microscope observations reveal that the aggregating material consists of spherical or discoidal particles of (flattened) diameter 380 Å. The aggregation pattern observed is that of microflocs.

7. The possible significance of these observations for the mechanism of the surface precipitation reaction and for sol-gel changes in the cell interior is discussed.

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A TIDAL RHYTHM IN BEHAVIOR OF MELANOPHORES IN AUTOTOMIZED LEGS OF UCA PUGNAX

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Studies of the behavior of marine animals have shown that a number of forms which normally are exposed to periodic tidal variations in their environment possess patterns of activity which are correlated with the tides. In some instances these tidal rhythms have been found to persist under constant conditions in the laboratory. The literature of this subject has been reviewed by Brown, Fingerman, Sandeen and Webb (1953).

The chromatophore system of the fiddler crab, *Uca pugnax*, has provided the basis for considerable study of rhythmic behavior. These crabs possess a diurnal rhythm of color-change, such that they are dark during the day, due to dispersed melanin within the chromatophores, and light at night, due to concentrated melanin. These diurnal variations are supplemented by a tidal rhythm of melanin dispersion possessing 12.4-hour cycles (Brown, Fingerman, Sandeen and Webb, 1953). The simultaneous possession of diurnal and tidal rhythms results in semilunar cycles of 14.8-day frequency, since it is at this interval that a given phase of tide coincides with a given time of day.

The daily pattern of oxygen consumption of *Uca* also shows variations which are tidally and diurnally rhythmic (Brown, Bennett and Webb, 1953). The diurnal rhythm in metabolic rate was demonstrated by determining the average oxygen consumption during each hour of the day of a 15-day period. The tidal rhythm was demonstrated by utilizing data for a 29-day period in such a fashion that the diurnal rhythm was randomized.

Both color-change and oxygen consumption are hormonally regulated processes. However, as yet there has been no direct demonstration of variations in the titer of hormonal substances in the blood of *Uca*. The chromatophores of legs autotomized by the crabs remain, at least initially, in contact with the blood-borne substances which must normally determine the degree of dispersion of the pigments. It was believed that the changes in the state of melanin dispersion following leg autotomy might reflect, in some fashion, the normal variations in hormonal concentrations which occur in the intact animal. The experiments to be reported here were begun in an attempt to determine what factors might be involved in the behavior of melanophores following autotomy.

MATERIALS AND METHODS

Uca pugnax were collected at Chapoquoit, near Woods Hole, Massachusetts, and maintained in the Marine Biological Laboratory in large white dish pans

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containing sea water to a depth of a few millimeters. The animals were maintained in continuous illumination which varied in intensity from 2 ft.c. during the night to approximately 15 ft.c. during the day. The stock group was replenished with freshly collected animals at intervals of two to four days. In no experiment were animals used which had been in the laboratory longer than four days.

In order to determine the behavior of the melanophores in autotomized legs animals were induced to autotomize two or three legs by applying pressure on a distal segment of the leg. The autotomized legs were placed in sea water in Syracuse watch glasses. In determining the degree of melanin dispersion characteristic of any group of legs the average index of the melanophores on the anterior surface of the meropodite of each leg was estimated, using the Hogben and Slome scale, in which 1 describes completely concentrated pigment, 5 describes completely dispersed pigment, and 2, 3, and 4 describe graded intermediate degrees of dispersion. The average of the indices estimated for the several legs of each group was considered to describe the degree of melanin dispersion characteristic of that group.

The experiments were conducted during the summer of 1953. The times of high and low tides referred to in this paper are those occurring on the day of the experiment at the site of collection of the animals.

EXPERIMENTS AND RESULTS

Series I. Characteristics of the behavior of melanophores during the hour following autotomy

The average rate of concentration in melanophores of fifty legs obtained from twenty-five animals selected randomly from the stock group was determined during the hour after the legs were caused to autotomize. The legs were divided into five groups, each consisting of two legs obtained from each of five animals. The average chromatophore index of each group was determined immediately following autotomy (10 A.M.), and again 10, 20, 30, 45, and 60 minutes following autotomy. The average chromatophore index of all legs at the time of autotomy was 4.7; the average values of the subsequent readings were 4.6, 4.3, 3.8, 3.5, and 3.5.

This procedure was repeated at 5 P.M. on the same day. The average chromatophore index of the fifty legs at the time of autotomy was 4.0. The average stages at 10, 20, 30, 45, and 60 minutes following autotomy were 3.7, 3.1, 2.9, 2.5, and 2.7, respectively.

The average chromatophore index determined for each group of ten legs at each reading is plotted in Figure 1. By calculating the slopes of the various intervals of each of these curves, it was determined that the maximum rate of concentration occurred during the first 30 minutes following autotomy in eight of the ten groups. The standard deviation of the 30-minute values obtained for the five groups of legs in the first experiment is ± 0.49, and that for the values obtained in the second experiment is ± 0.33.

It was concluded that in frequent determinations of the behavior of the melanophores in autotomized legs, the average chromatophore stage of a group of ten legs determined 30 minutes after autotomy could be used as a good index of the direction and the relative rates, and total amount of change in the melanophores. Numerous additional observations have also indicated that the chromatophore stage observed

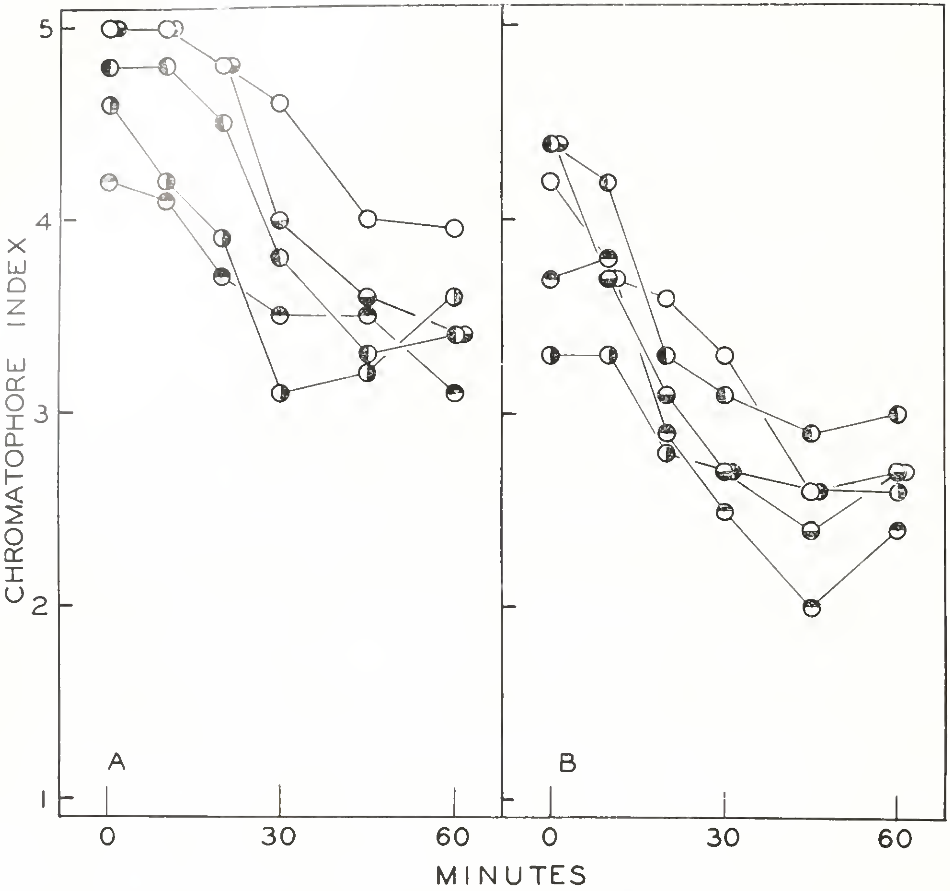


FIGURE 1. The behavior of melanophores of *Uca pugnax* during the hour following autotomy (Series I). The values for the five groups of legs autotomized at 10 A.M. are represented in the A portion of the graph, while those for the legs autotomized at 5 P.M. are given in the B portion.

30 minutes following autotomy is as accurate an index of such melanin changes as one could find.

Series II. Behavior in legs with maximally dispersed melanin

Crabs maintained in an illumination of 100 ft.c. were used in preliminary studies of factors affecting the melanophore behavior in autotomized legs. For each determination two legs were removed from each of six animals in which the melanin was maximally dispersed, and the average chromatophore index of the groups was determined 30 minutes after autotomy. A total of 32 determinations were made between 5 A.M. and 10 P.M. on 7 days.

These data are summarized in Figure 2. The difference between the time of day at which autotomy was induced and the time of day of the nearest high tide was calculated for each determination; the average chromatophore index observed 30 minutes after autotomy has been plotted against the difference so obtained. The lowest 30-minute values, indicative of the greatest concentration, were observed when the determinations were made near the time of high tide. The highest 30-minute values were observed when autotomy was induced between the times of a high tide and a low tide.

Series III. Relationship between the time of high tide and the behavior of melanophores

In order to determine any variation in melanin concentration which might be correlated with the time of day at which high tide occurs, observations were made through the daytime over a period of 23 days. Crabs of the stock group maintained in continuous illumination of 2- to 15-ft.c. intensity were used in this study. Autotomy of two legs of each of five animals, selected randomly, was induced at half-hour intervals. The average melanophore stage of each group of legs was deter-

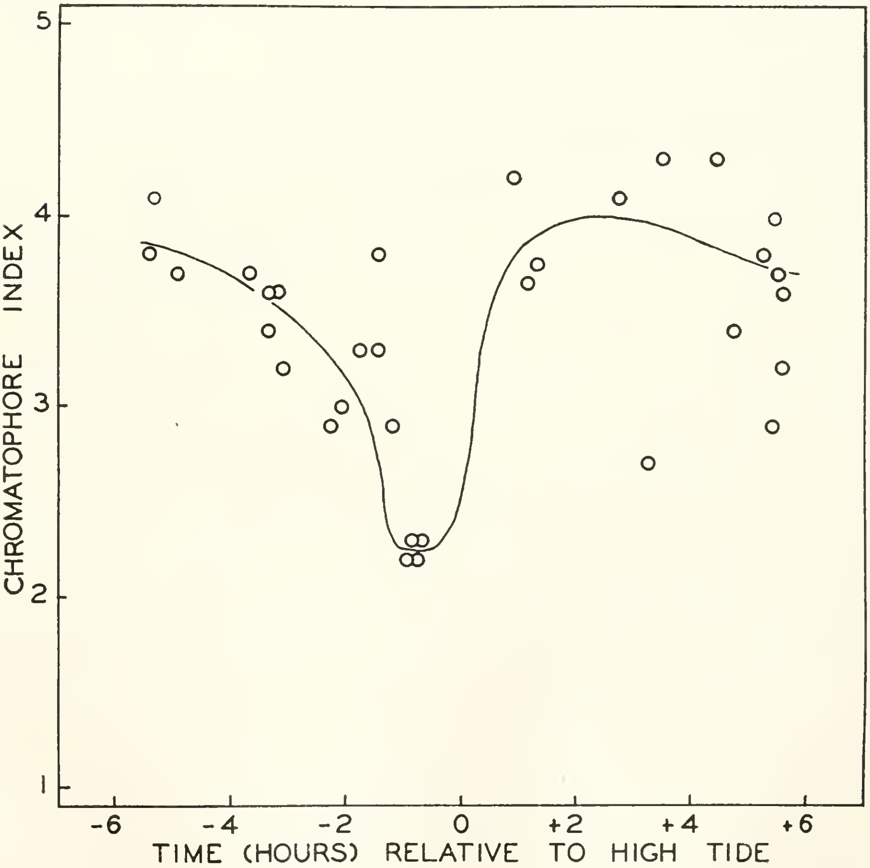


FIGURE 2. The 30-minute values in legs autotomized by animals with maximally dispersed melanophores (Series II). The values are plotted against the difference between the time of day at which autotomy was induced and the time of day of the nearest high tide.

mined at the time of autotomy and again 30 minutes afterwards. During the first few days of the series observations were made between 8 A.M. and 5 P.M. During the latter portion of the series observations were extended to include earlier and later periods.

In analyzing these data the initial melanophore indices of the two groups of legs studied in any one hour were averaged to give a measure of the average dispersion of melanin for the population at that time. Also the 30-minute values obtained for the two groups observed during each hour were averaged to give a measure of the characteristic melanophore stage at the end of the half-hour following autotomy at that time of day. The differences between these average initial values and their respective 30-minute values provide the basis for calculating the rates of concentration characteristic of each hourly period.

The behavior of melanophores of legs obtained from this stock population clearly reflects the diurnal rhythm of color-change of the intact animals. In only a few cases did dispersion of melanin occur following autotomy. Consequently the low values characteristic of the intact animals during the periods of transition between day and night phases are consistently associated with low 30-minute values.

The basic diurnal pattern in behavior of melanophores can be demonstrated by determining the average value for each hourly interval for a 15-day period. The tidal rhythm, which also is significant in this series, is thereby randomized, since each phase of the tidal cycle will occur once in relation to each hourly determination during a 15-day period. This procedure was employed in determining the diurnal pattern illustrated in Figure 3. The behavior following autotomy (Curve B, which represents the 30-minute values) clearly reflects the diurnal rhythm of the intact animals seen in Curve A, which represents the initial values. In addition, there appears to be a diurnal rhythm in rate of concentration, as indicated by the increased width of the band bounded by the two curves during the afternoon hours.

The tidally rhythmic behavior in this series can best be seen by examining the data for individual days. These are presented graphically in Figures 4 and 5. Since a detailed analysis of the normal daily variations in melanophore dispersion has been made by Brown, Fingerman, Sandeen and Webb (1953), the Curve A for each day, describing the degree of dispersion at the time of autotomy, will not be analyzed here. These curves are included only to indicate the "baselines" for the changes occurring in the autotomized legs. However, the daily character of the behavior of the melanophores following autotomy, as described by the series of 30-minute values for each day (Curve B), will be considered in some detail.

Examination of the series of 30-minute values observed on August 10, 11, 12, 13, 14, and 15 (Fig. 4), shows that high tide occurred within an hour of the time at which a minimum of melanin dispersion was seen. On these days high tide occurred between 8 A.M. and 12 noon.

The observations on August 17 to 22, inclusive, are also presented in Figure 4. On August 17 the lowest in a series of generally low values was seen at the usual time relative to high tide. On August 18, four values obtained around the time of high tide formed a broad minimum, but again the lowest value was seen near the time of high tide. Any increase in concentration correlated with the high tide which occurred at 3 P.M. on August 19 was more or less masked by the generally increasing degrees of concentration which occurred during the afternoon.

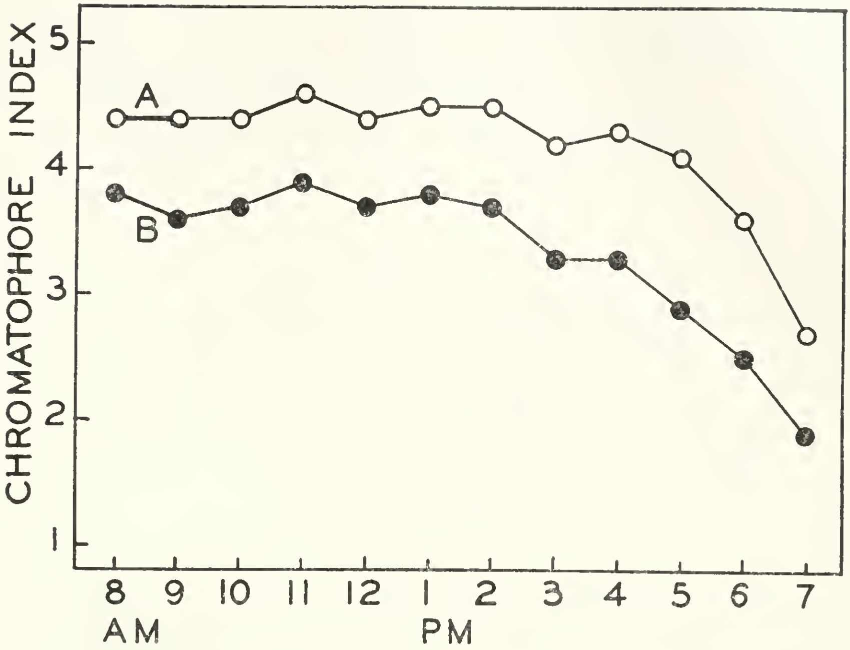
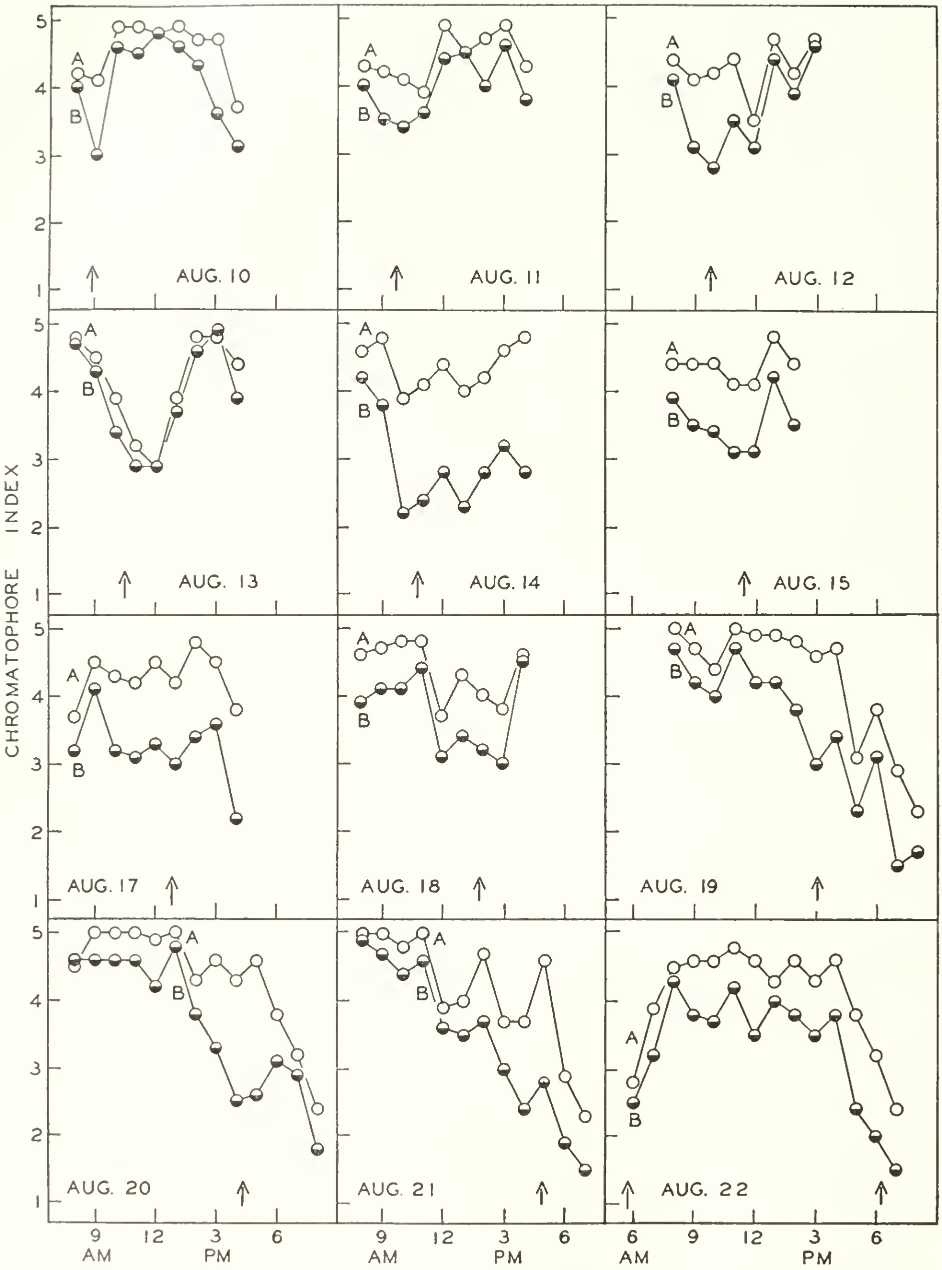


FIGURE 3. Diurnal variation in behavior of melanophores in autotomized legs, obtained by averaging data from August 18 through September 1, inclusive (Series III). Open circles (Curve A) represent the average values at the time of autotomy, while solid circles (Curve B) represent the average values 30 minutes after autotomy.

On August 20 a minimum value occurred at the time of high tide. The degree of dispersion reached, however, an over-all minimum at 8 P.M. in the diurnal rhythm of change. On August 21, when high tide occurred at 4:50 P.M., the tidal chromatophore change was obscured by the diurnally rhythmic change. The observations of August 22 were begun just following the time of high tide at 5:40 A.M. A rapid increase in degree of dispersion was observed, and was found to be maintained until shortly before the time of the evening tide.

On August 23, 24, and 25 (Fig. 5) observations spanned the time of both morning and evening high tides. In general, the increase in concentration typical of the time of high tide appeared to be obscured by the simultaneous occurrence of the transition between the night and day phases. The expression of the tidal rhythm was apparent chiefly as the delayed occurrence of strong dispersion during the morning, and the early appearance of concentration during the afternoon. On August 24, however, a slight but nonetheless definite increase in concentration was correlated with the time of the high tide which occurred at 7 A.M.

Figure 5 also includes the observations from August 26 to September 1, inclusive. An increase in concentration was associated with the time of high tide on August 26 and again on August 27. On August 28 this characteristic tidal response, although somewhat obscured by the low degree of dispersion observed throughout the day, was again apparent. On August 29 progressively greater degrees of concentration were observed following the 7 A.M. determination, reaching



FIGURES 4–5. The melanophore behavior in the autotomized legs of crabs studied in Series III. Open circles (Curve A) represent the values observed at the time of autotomy and half-closed circles (Curve B) represent the values observed 30 minutes following autotomy. The times of high tides are indicated by arrows.

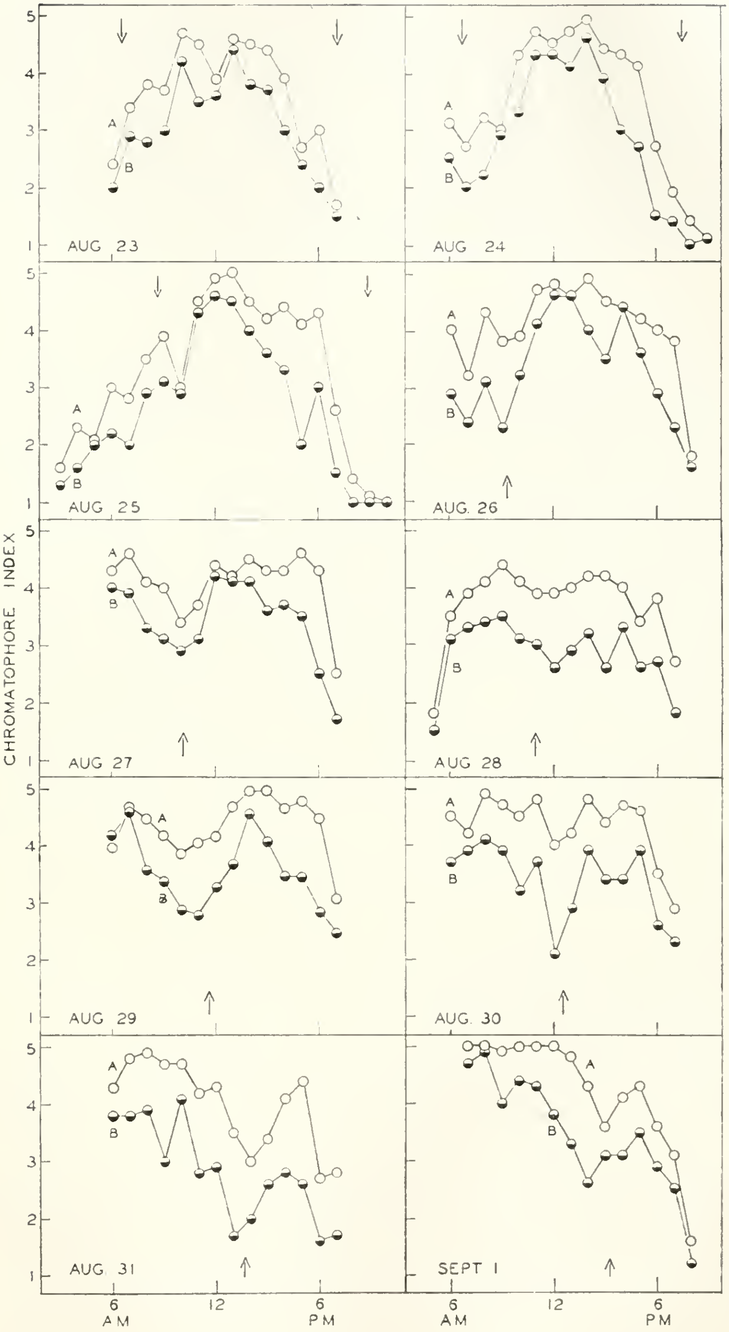

CHROMATOPHORE INDEX
1
2
3
4
5
AUG 23
AUG 24
AUG 25
AUG. 26
AUG 27
AUG 28
AUG 29
AUG. 30
AUG. 31
SEPT 1
A
B
6 AM
12
6 PM

FIGURE 5.

a minimum correlated with the time of high tide at 11:30 A.M. A second peak occurred three hours after the time of high tide; subsequently, increasing degrees of concentration correlated with the onset of the night phase were observed.

Between 6 A.M. and 5 P.M. on August 30 there was a sustained tendency toward dispersion, with the exception of the values obtained near the time of high tide at 12:30 P.M. The characteristic increase in concentration was seen at the time of high tide on August 31. On September 1 an increasing degree of concentration was observed throughout the observations, but the minimum of the day phase values was seen during the hour preceding the time of high tide at 3 P.M.

DISCUSSION

The behavior of melanophores of legs autotomized by light-maintained animals has been shown to be characterized by varying degrees of pigment concentration. The extent of concentration, as measured 30 minutes following autotomy, reflects the diurnal change in pigment dispersion which occurs in a population maintained in continuous illumination of low intensity (Series III). During the night phase, and the periods of transition between night and day phases, the relatively concentrated state of the melanin of the intact animals serves as one limiting value on the range of change in melanin dispersion in isolated legs. However, the relationship between the initial chromatophore values and the 30-minute values is not a parallel one. When the tidal influences are removed by averaging the data of one tidal cycle, it becomes apparent from the width of the band bounded by Curves A and B, Figure 3, that an increased rate of concentration is characteristic of afternoon and early evening determinations. The average rates of concentrations, expressed as chromatophore-unit change during the 30 minutes following autotomy, range from 0.6 for 8 A.M. to 1.2 for 5 P.M. Thus two-fold differences may exist between the behavior characteristic of the morning and afternoon hours, other factors equal.

Although there are insufficient data to exclude tidally rhythmic influences, examination of the available data suggests that lower rates of concentration are characteristic of the later evening hours. The relationship prevailing during the early morning hours, when the melanin of the intact animal is dispersing, remains obscure. It can be seen from the foregoing that the 30-minute values reflect not only the diurnal rhythm in the melanophores of the intact animal, but also a diurnal rhythm of ability to concentrate following autotomy, with the forms of these two rhythms differing slightly from one another.

Superimposed on the diurnal rhythm evident in the 30-minute values are modifications which are correlated with the phases of tidal cycles. The expression of these modifications is a function of the time of day at which the various tidal phases appear. Correlated with low tides occurring between 6 A.M. and 4 P.M. there is a characteristic high degree of dispersion in the isolated legs. In the evening this low-tide phase is antagonized by the diurnally rhythmic mechanism to produce both lessened dispersion of the pigment of the intact animals, and increased rate of concentration following autotomy. In contrast, a low degree of dispersion following autotomy near the time of high tide is characteristic whether or not the diurnal tendency for dispersion in the intact animals and maintained dispersion of melanin in isolated legs is present. During the day phase the degree of dispersion observed 30 minutes following autotomy reaches a minimum near the time of high tide, thereby providing a method whereby the phase of the tidal rhythm of the crabs may be determined, as described in a preliminary report of these experiments (Hines, 1953).

Although in many cases a lower initial degree of dispersion can be observed around the time of high tide, the increased amount of concentration following autotomy that is associated with the time of high tide is not simply a reflection of the degree of dispersion at the time of isolation. There is a general tendency toward an increased rate of concentration near the time of high tide. This is readily apparent from Series II, and is also evident in the results of Series III (see especially the data for August 10, 12, 20, and 30).

The isolated-leg preparation provides a method for following changes in chromatophore stage resulting from the gradual inactivation of the hormonal substance or substances responsible for the stage of the chromatophores in the intact animal. It may be considered as a system in which the chromatophores remain in contact with a medium containing hormonal substances, but a medium into which the normal centers of hormone production no longer secrete additional chromatophorotropins.

Sandeen (1950) has demonstrated that a melanin-dispersing substance is present in extracts of both the sinus gland and central nervous organs. If only one such substance were responsible for the state of the chromatophores, one would expect that the various degrees of dispersion in the intact animal would be achieved by means of varying concentrations of this one substance in the blood. In this case, one would also expect that the rate of inactivation of the substance would be a function of its concentration and that, following autotomy, a specific rate of pigment concentration would be associated with each particular degree of initial dispersion. If such a simple relationship between initial degree of dispersion and rate of concentration following autotomy is not observed, it would appear that something other than a single dispersing hormone is operative in determining the state of the melanophores. The observations of Series II are illustrative in this regard. In every case, the melanin was maximally dispersed at the time of autotomy. However, the extent of concentration which occurred during the 30 minutes following autotomy varied widely.

The foregoing evidence for a dual or multiple humoral control of the melanophores is substantiated by the overlapping ranges of the 30-minute values of Series II and III. In Series III the initial intermediate degrees of dispersion were often associated with 30-minute values greater than the lowest 30-minute values observed in Series II, indicating that the various rates observed in Series II do not reflect simply inactivation of various amounts of dispersing hormone in excess of that which can be reflected in the average stage of the melanophores.

It would appear, then, quite difficult to interpret these data in terms of the action of a single dispersing hormone. The presence of a melanin-concentrating principle was first postulated by Brown and Webb (1948), and was apparently required for an adequate interpretation of the responses of *Uca* to changes in illumination (Brown and Hines, 1952) and to photoperiod (Brown and Stephens, 1951). These experiments were interpreted in terms of the role of both a dispersing and a concentrating hormone in determining the normal day-to-night and night-to-day changes. Subsequently, the degree of pigment dispersion during the day phase was found to be correlated with the phases of the tidal rhythm (Brown, Fingerman, Sandeen and Webb, 1953). The present study of the behavior of melanophores in autotomized legs suggests that the ratio of effective concentrations of dispersing and concentrating hormones must be influenced by the tidally rhythmic mechanism, as well as by the diurnally rhythmic one. If two antagonistic hormones are responsible for determining the initial degree of dispersion, it becomes possible for any given intermediate degree of dispersion to be the result of a wide range of concentrations of one of the substances in the presence of directly varying amounts of a second. The diurnal and tidal variations in rate of concentration following autotomy may then be considered as a reflection of rhythmic variations in the amounts of the two substances. It would also appear that there is a more rapid loss of dispersing than of concentrating activity.

SUMMARY

1. The melanophores of the legs of *Uca pugnax,* following autotomy induced during the day phase of the diurnal rhythm, tend to concentrate their pigment. An adequate measure of the extent of concentration characteristic under any given conditions can be determined by observing the degree of dispersion 30 minutes following autotomy.

2. The degree of dispersion measured 30 minutes following autotomy reflects both a diurnal and a tidal rhythm in the behavior of melanophores in autotomized legs. During the day phase, when the melanin of the intact animals is dispersed, relatively little concentration occurs following autotomy near the time of low tide. An increased degree of concentration is observed when legs are isolated near the time of a high tide occurring during the day phase. This tidally rhythmic behavior has been observed for both a population with maximally dispersed melanin, and for one with normally varying degrees of melanin dispersion.

3. The variations in rate of concentration which have been observed cannot be explained as due solely to the inactivation of the melanin-dispersing hormone. It is necessary to assume that both the state of the melanophores of the intact animal and the character of the response following autotomy are determined by the relative concentrations in the blood of a melanin-dispersing hormone and a melanin-concentrating hormone.

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THE INFLUENCE OF SEASONAL ENVIRONMENTAL CHANGES UPON THE METABOLISM, LETHAL TEMPERATURE AND RATE OF HEART BEAT OF GAMMARUS LIMNAEUS (SMITH) TAKEN FROM AN ALASKAN LAKE

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Fresh water biological studies in Alaska have until quite recently been conducted mainly by scientists visiting the Territory during the few summer months. Consequently very little has been known about the conditions under which the fresh water fauna exist throughout the seasons. Prolonged winter cold, brief summer warmth and the restrictions of the habitat caused by the dehydrating effect of extensive winter freezing are environmental factors not encountered by aquatic life in temperate regions. These conditions, however, bring out a number of problems of considerable basic scientific interest.

It has therefore been the plan of this study to examine the metabolic rate of the amphipod crustacean *Gammarus limnaeus* (Smith) for evidence of adaptive adjustments to the extreme seasonal changes typical of Alaska for comparison with the adaptations to temperature shown by aquatic animals from other regions. *Gammarus limnaeus* (Smith) was selected as a suitable subject for this study because of its wide distribution in Alaska, its importance as a fish food, and its consequent significance as a link in the chain of reproduction in fresh water. Its close taxonomic relation to other amphipods abundant in much of the northern world facilitates comparison.

Gammarus limnaeus (Smith) belongs to the genus *Gammarus* (Fabricius) and the amphipod family Gammaridae. The genus is very widely distributed in both salt and fresh water. Over 30 species are known, and of those 6 are found in North America, *Gammarus limnaeus* together with *Gammarus fasciatus* being the most common and widely distributed of the northern species; both occur in Alaskan lakes and ponds. *Gammarus limnaeus* is found throughout the United States from Maine to New Mexico (Weckel, 1907). Related species are common in fresh water throughout Siberia and Northern Europe.

PREVIOUS INVESTIGATIONS

The underlying proposition for this study was first expressed by Krogh (1916, p. 101): "One would expect that animals living at a very low temperature should show a relatively high standard metabolism at that temperature compared with others living normally at a high temperature." The abundance and activity of life in cold northern waters in which poikilotherms from warmer waters would be injured or slowed to ineffectiveness by cold is in good agreement with this basic statement.

Considerable information is available on the reaction of poikilotherms to changes in temperature and oxygen tension during experiments in the laboratory. Less attention, however, has been given to the matter of determining 1) whether certain physiological adjustments of transitory or more permanent nature take place in cold blooded animals when in their natural surroundings, and 2) to what extent these adjustments might help the animal overcome changes in the environment that otherwise could have an adverse effect upon its success in living in the habitat.

Although only a few investigations seem to have been specifically directed toward the detection of seasonal or local variation in the metabolism of aquatic poikilotherms, it is not uncommon to find references to such differences in the temperature-metabolism relation of cold blooded animals. Cronheim (1911) considered that analysis of Knauthe's (1898) experiments with carp showed seasonal variation in metabolic rate. Wells (1935) found that the Pacific killifish (*Fundulus parvipinnis*) showed variations in its metabolic rate during the year and connected the fluctuations with the yearly changes in temperature of the waters in which they lived. Bruce (1926) carried out a year's study on *Mytilus edulis,* and found a seasonal variation in the oxygen requirement of this species which he associated with the reproductive cycle. Haugaard and Irving (1943) found such small seasonal variations in the oxygen consumption of the cunner (*Tautogolabrus adspersus* Walbaum) that the slightly higher oxygen consumption in winter than in summer at each temperature below 15° C. appeared too small to be regarded significant as an adaptation. In the sand crab (*Emerita talpoidea* Say), Edwards and Irving (1943) found a change in the relation of oxygen consumption up to four times higher in winter at 30° C. than in summer. The higher oxygen consumption rates in winter they interpreted as an adjustment by means of which the animals sustained activity and growth at the low winter temperatures.

Variation in the metabolism and such related functions as heart beat and pleopod movements in animals apparently identical systematically but taken from different latitudes has been reported by several authors, *viz.* Fox (1936, 1938, 1939), Fox and Wingfield (1937), Wingfield (1939), Spärck (1936) and Thorson (1936). The general assumption has been that the different temperatures of the two environments are responsible for the differences observed between the two groups in physiological response to changes in temperature during the experiment.

Marshall, Nicholls and Orr (1935) found seasonal differences in heat tolerances of *Calanus finmarchicus* with elevation of the lethal temperatures from 24° C. winter to 26° C. summer.

Hörstadius (1915) noted the point of normal development for *Paracentrotus lividus* eggs and found it to vary with the season. Runnström (1936) noted similar adjustment of both the upper and the lower limits for the development of the tunicates *Ciona intestinalis, Phallusia mammillata* and *Ascidia mentula,* and the sea urchins, *Paracentrotus lividus* and *Arbacia aequituberculata.* Fry (1947) in his paper on the effects of the environment on animal activity gives an extensive review of the existing literature in this field, in which he points out the importance of considering the environmental factors in physiological research and discusses the problems of acclimatizations.

Scholander, Flagg, Walters and Irving (1953) reviewed the literature of adaptation to cold and from their own observations compared the metabolism of arctic and tropical animals, including in their list terrestrial as well as aquatic poikilothermic forms. A general summary of their findings was that arctic aquatic forms in their accustomed temperature near 0° C. maintained a metabolic rate $\frac{1}{3}$ to $\frac{1}{10}$ as great as the tropical forms in their usual temperature of 30° C. If, however, the metabolic curve of the tropical forms is extrapolated down to 0° C. it represented only $\frac{1}{30}$ to $\frac{1}{40}$ of their metabolism in their tropical environment. Arctic forms showed a considerable, although not complete, adjustment of metabolism to their environment. In terrestrial forms, on the other hand, practically no such adjustment appeared (Scholander *et al.*, 1953).

The previous findings and reports thus all indicate the ability of aquatic poikilotherms to adjust themselves to their surroundings and also show the necessity for taking into consideration the original environment of the test animals in physiological experiments. From these former findings it could be expected that the amphipods from an Alaskan lake would show a higher oxygen consumption at a given low temperature in winter than in summer, with a higher metabolism that could facilitate a higher rate of growth in the winter months than would be expected from their temperature-metabolism relation in summer. In order to regard physiological processes as adaptive it is of prime importance to refer them to the conditions of the animal's natural environment and their behavior during the cycle of the seasons.

MATERIAL

The animals used in the experiments were all taken from Goose Lake, an eutrophic-type lake about two miles south of Anchorage, Alaska, averaging about eight feet in depth and covering about 30–40 decares. The margins of the lake are swampy, and there is no definite inlet or outlet. The banks contain deposits of peat over gravel and the bottom of the lake consists of a $1\frac{1}{2}$-foot deep layer of mud and decomposing material, a so-called false bottom underlain by a base of glacial gravel. In summer the lake has a rich vegetation of water lilies, *Nymphaea*, and other water plants, with water temperatures rising to more than 20° C. In winter the lake freezes to a depth of about 3–$3\frac{1}{2}$ feet or more. It is doubtful, however, that it ever freezes to the bottom. Ice is on the lake from the middle part of October to the first part of May.

The animal life of the lake is surprisingly rich, with many insect larvae and a number of crustaceans including many cladocera and smaller numbers of amphipods and copepods. Snails, leeches, flatworms, and mites are also common. In winter the amphipods seem more abundant and a smaller number of cladocera and copepods are present. Whirligig beetles (Gyrinidae), boatmen (Corixidae), and water beetles (Dysticidae) are found as adults, and snails, flatworms, leeches, and the larvae of dragonflies (Aeshnidae), and damselflies (Agrionidae), are commonly found on the bottom.

Only one species of amphipod was found, *Gammarus limnaeus* (Smith), described by S. I. Smith (1874). The animals were present in the lake in fairly large numbers throughout the year. The main breeding season of *G. limnaeus* seems to be shortly after breakup of the ice in the spring. At that time they are abundant along the shores, where many are found copulating in the shallow water. Some ice may still remain in the middle of the lake. The number of *G. limnaeus* seems to fluctuate through the summer, varying according to location.

The *G. limnaeus* used for study were caught with a dip net from the shore or from a rubber raft in summer, and in the winter through a three-foot square hole in the ice which was allowed to freeze over to keep the water from contact with air. In the laboratory the amphipods were kept in large thermos jars at as near the lake temperature as possible. Animals were usually caught in the morning and observations were started immediately, thereby avoiding prolonged exposure to indoor conditions and significant changes in temperature. The animals used were of various sizes and of both sexes as no significant sex differences in oxygen consumption were observed. Animals infested with parasites, such as acanthocephalid larvae and what was probably a larval tapeworm, were eliminated. All animals carrying eggs or showing low vitality were discarded.

EXPERIMENTAL PROCEDURE

1. O_2 consumption. Oxygen consumption was measured by means of Scholander's micro- and semi-micro respirometers (Scholander, 1950). The temperatures in the water baths were kept constant within plus or minus 1° C. During most of the experiments simultaneous runs at four different temperatures, ranging from 0° C. to 22° C., were performed so as to utilize animals with as similar physiological background as possible. Fifteen individuals in separate chambers were used at each temperature and necessary handling was done by means of a pipette so as to avoid damaging the fragile legs or respiratory organs. Filtered lake water was used in the respiratory flasks. Blanks with lake water were run parallel to the experiments in the same water bath. Measurements were timed from several hours up to 24 to secure representative data for the oxygen consumption. Day and night measurements and those conducted inside a dark, walk-in ice box revealed no significant diurnal cycles or influence of light. Data obtained on animals that died during one observation and on those showing low vitality were discarded.

2. Adequacy of oxygen supply. Consideration was given to the possible influence of changing oxygen tension upon the metabolic rate of invertebrates, as reported by Amberson, Mayerson and Scott (1924). Analysis of the water from the vials after the termination of the measurements revealed that the animals themselves, by their respiratory movements and activity, stirred the water sufficiently to keep the oxygen content of the shallow water in the vials at more than $\frac{2}{3}$ saturation. Analysis of $\frac{1}{2}$ cm.^{3} of the water from the respiration vessels was made by means of the Winkler method modified according to van Dam (1935), and conducted inside a syringe. Titration was carried out with Scholander's microburette (Scholander, Edwards and Irving, 1943), using a magnetic stirring device operating as described by Linderström-Lang and Holter (1940).

3. Weight and composition of specimens. After the runs the animals were blotted with a filter paper and dried to constant weight without heat in a desiccator with calcium chloride, to determine the water content and the dry weight of the animals. A determination of the hydrolyzable material of the animals was carried out to facilitate comparison with similar figures cited in the literature. For this purpose a group of fifty animals was dried with filter paper, weighed, and dried in a desiccator in the same way as for determination of dry weight. After weighing, the animals were boiled in 10% KOH for one hour and the residual matter filtered, dried and weighed. Residue, principally chitinous and similar materials undissolved in KOH, might be considered inert in ordinary respiration. The weight of

TABLE I

Content of water and hydrolyzable material of Gammarus limnaeus (*Smith*)

Date	Wet wt. of 50 animals grams	Dry wt. of 50 animals grams	Water %	Hydrolyzable material of 50 animals grams	Hydrolyzable material of 50 animals %
1–9–51	1.020	0.240	79.6		
1–15	1.010	0.215	78.8		
1–23	1.020	0.225	77.9		
3–12	0.860	0.165	80.8	0.816	14.0
3–26	0.782	0.143	81.8		
3–28	0.850	0.160	81.2		
4–2	0.922	0.167	81.8		
4–4	0.927	0.165	82.2		
4–9	1.076	0.184	82.9	1.016	11.5
4–12	0.994	0.174	82.5	0.938	11.8
4–16	0.975	0.165	83.1		
5–7	0.948	9.194	79.5		
5–10	0.972	0.194	80.1		
5–16	1.067	0.199	81.3		
5–24	1.057	0.214	79.8		
6–7	1.126	0.233	79.3		
6–11	1.035	0.195	80.1		
6–21	1.465	0.265	81.9		
7–10	1.025	0.207	80.3		
9–20	1.110	0.220	80.2		
9–26	1.060	0.215	79.7		
10–8	1.237	0.240	80.8		
10–22	1.635	0.320	78.6		
11–21	1.412	0.315	79.1		
12–6	1.390	0.257	81.5		
12–13	1.750	0.352	79.8		
12–20	1.557	0.332	78.5		
1–9–52	2.020	0.410	79.7		
1–17	1.377	0.281	79.5		
2–5	1.698	0.349	79.4		
4–8	1.812	0.287	84.3		
Average			80.5	0.923	12.4

the hydrolyzable material was calculated in per cent of wet and dry weight (see Table I). There was no suggestion that this residue changed sufficiently to alter results. The reference of oxygen consumption to wet weight, dry weight or hydrolyzable material should therefore give about the same relationship. The oxygen consumption was most conveniently given per unit wet weight and was chosen as a basis for the figures used in the accompanying tables and graphs.

4. "Lethal" temperature. Determinations of the "upper lethal" temperature at the end of the winter and in the summer were made. The highest tolerable temperatures were determined by suspending small glass vials containing lake water and 5 animals each in thermos bottles filled with water with a temperature difference of 2° C. from 24° C. to 36° C. The vials were checked every 15 minutes and the dead animals were picked out. Paralyzed animals showing no signs of life were counted together with the dead ones even though a few recovered when placed in cold water. Any attempt to distinguish between paralyzed and dead animals would be very difficult and possibly irrelevant. One hundred per cent survival after one hour exposure to a given temperature was used as a standard for determining the animal's level of heat tolerance.

5. Heart rate. The heart beat was counted by means of transmitted light that had passed through a ¾-inch thick layer of flowing water in a specially constructed water-cooled microscope stand (Krog and Simmet, 1954) which permitted temperature control to within plus or minus 0.1° C. The animals were surrounded by lake water during the test. The temperature of the water immediately surrounding the animals was measured by means of a fine thermocouple. Four to five ½-minute counts were taken at each temperature and the average rate of heart beat calculated.

RESULTS OF OBSERVATIONS AND EXPERIMENTS

Temperature and oxygen supply in the lake. The curves in Figure 1 show the yearly variation in temperature and oxygen content of the water in Goose Lake, together with the saturation curve for oxygen dissolved in the water at the recorded temperatures. The oxygen content in the lake reached its lowest values in February and March when it dropped below amounts detectable with the Winkler method used for this determination (Standard Methods for Examination of Water and Sewage, page 724). The summer months give quite steady values for dissolved oxygen. As seen from the curve, the increase of oxygen in the lake starts before the breakup of the ice. This is probably due to accumulation of water from melting snow on the top of the ice, which is later drained into the lake through enlarged cracks. This incident is followed by a lifting of the ice caused by the rising water level and subsequently by appearance of open water along the shores. From this time on, direct contact between the lake water and the air is established, and the O_2 content increases gradually.

The water in the lake, however, never reached saturation at any temperature. After the oxygen content reached a value of around 8–9 ppm., it leveled off and remained at that level throughout the summer. The first decrease of oxygen in the lake followed, after some delay, the freeze-up in the fall and it continued to decrease at a fairly even rate in the following months until it reached its minimum in February, March, and April. Reduction of the oxygen supply in winter is probably common in the shallow lakes of Alaska and might explain why no fish have been found in so many of the smaller, shallow, non-circulated lakes although fish food appears to be abundant.

The temperature of the water in the lake also varied considerably with the season, rising in summer above 20° C. (see Fig. 1). The temperature increased rather sharply in the spring because of the shallowness of the water and the lack of any significant out- and in-flow. The pH was not observed to change consistently with the season and stayed between 7.0 and 8.0.

Variation of the amphipods' oxygen consumption with size was found to occur in the course of the year as is shown when the oxygen consumption at 10° C. is plotted against weight (see Fig. 2). Curves plotted at other temperatures show similar although not so pronounced variation. A further breakdown of the data in weight groups was not found desirable as it would complicate the final comparison between summer and winter animals and only slightly improve the spread of the values.

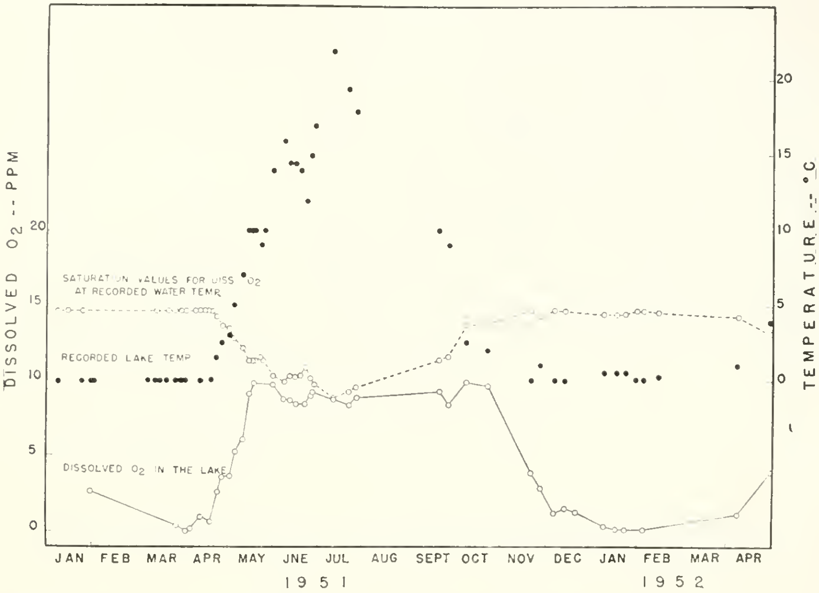

FIGURE 1. Recorded temperature in Goose Lake 1951 through April 1953, together with calculated saturation values for dissolved oxygen at those temperatures, and actual recorded dissolved oxygen during the same period, measured in parts per million by weight.

To simplify comparison, the year was divided into two seasons—June and July—(see Fig. 3) representing the typical summer period with fairly high temperatures and rather constant oxygen content of the water, and the late winter—February through March—which is the period of lowest oxygen content, disregarding the transition between these two periods. During the winter period a significant lowering of the oxygen utilization of the animals was found to occur when the oxygen content in the lake water was lowest, suggesting a difference between the seasonal adjustment in these animals and the Crustacea previously investigated. These have, however, been mainly marine forms or those from well circulated waters with fairly high and stable oxygen tensions and with the temperature as the most important variable.

Figure 4 shows the upper temperature tolerance as determined for the animals in winter as well as in summer. The highest temperature which the animal can tolerate as determined by 100% survival for one hour, seems to be about 26° C. for the winter animals, and for the summer animals from 30°–32° C. This shows a lowering of the resistance to heat by 4°–6° C. in the cold season as compared to the summer, which is about what would be expected from previous investigations of temperature adjustments in invertebrates.

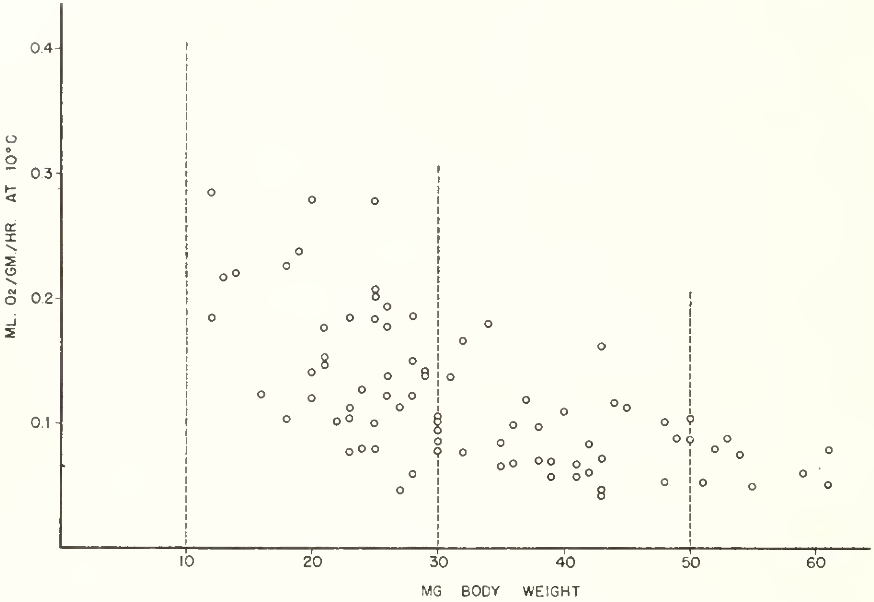

FIGURE 2. Relation between body weight and oxygen consumption at + 10° C.

The pulsation rate of the "heart," or more correctly the dorsal vessel of the animal, was recorded at the end of winter (March) and at the end of summer (the last part of August) in order to determine the extent of seasonal variation. Figure 5 shows the rate of pulsation plotted against temperature. A slight elevation in the relation of heart rate to temperature seemed to occur in winter with generally lower heart rates at all temperatures for the summer animals. Above 19° C. the winter animals were observed to show irregularity in their "heart" beat with an eventual decrease in rate. The larger spread in the data for the winter animals at the higher temperature as compared with the summer animals is believed to be mainly caused by this reaction (see Fig. 5). The summer animals on the other hand showed a normal increase of the "heart" beat above 14° C. up to around 23° C. after which irregularity and consequent gradual drop in heart rate occurred as in the winter animals.

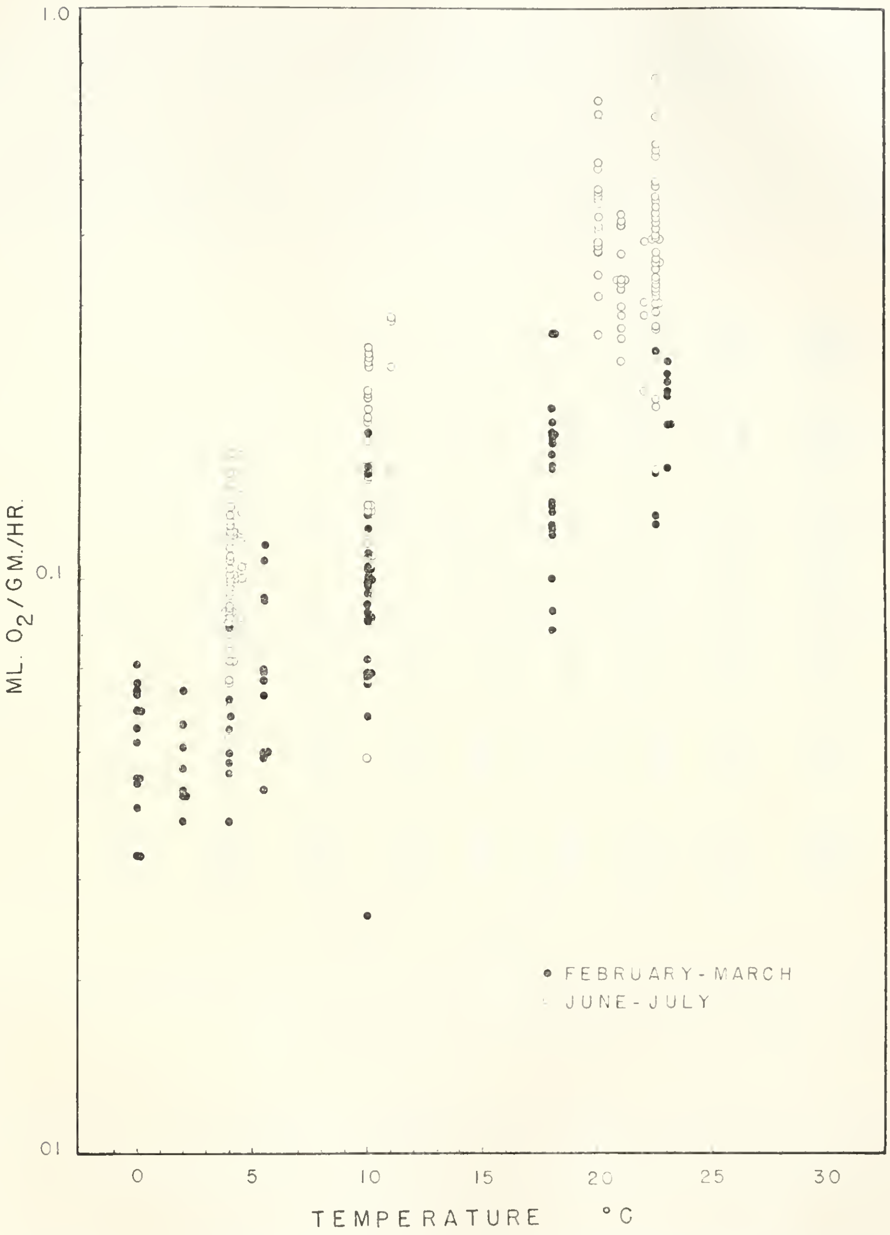

FIGURE 3. The relation between oxygen consumption and temperature in the periods February through March and June through July, plotted on a semilog scale. The animals from the two different seasons are marked with separate symbols.

Discussion

On the basis of previous studies of the adaptation of the metabolism of aquatic invertebrates to temperature, it was anticipated that animals from an Alaskan lake, with the relatively long exposure to low water temperatures, would show a metabolic adjustment so as to consume more oxygen at a certain low temperature in the winter than at the same temperature in the summer. No such adjustment has been observed in the present study. On the contrary, a decrease in oxygen consumption in February and March can be noticed (see Fig. 3). Although the variation is not large the constancy and trend of the changes indicate their significance. It will therefore seem natural to connect the low oxygen consumption of the amphipods in late winter with the low oxygen content of the water at that time. Pursuing the same reasoning, the absence of the expected adaptation in metabolic rate to low winter temperatures, as found by investigators in other situations, could be caused by the fact that there is not enough oxygen for such an adaptation to be useful.

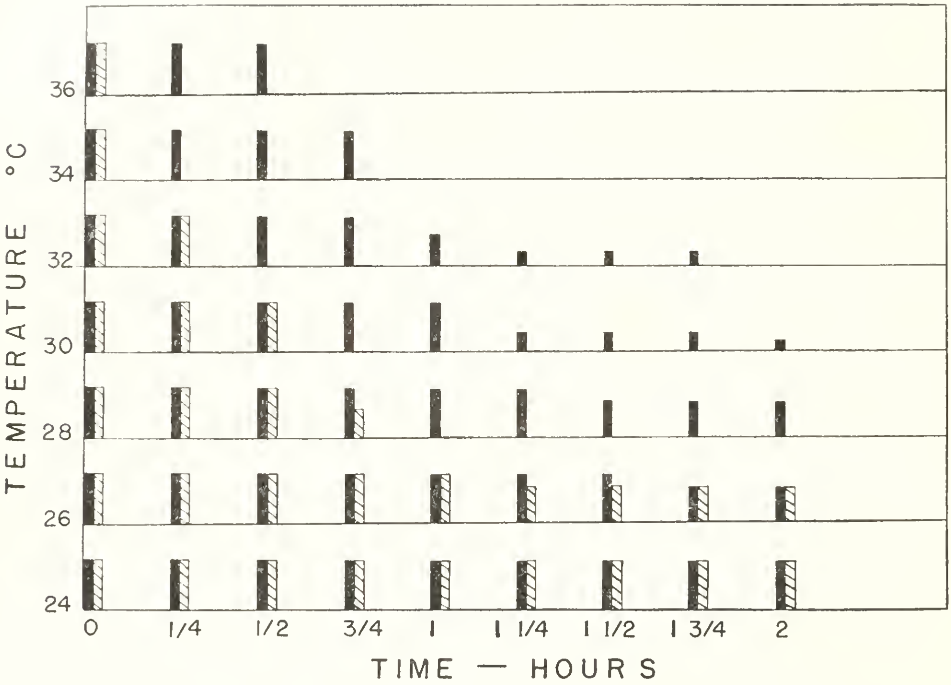

Figure 4. Diagram shows upper lethal level of the amphipods respectively in the winter and in the summer. Full length columns at extreme left represent five animals each. Filled columns are summer animals, cross lined are winter animals.

Assuming that adjustments made by animals in the face of unfavorable environmental factors are directed toward the factors that are most critical, the differences between previous findings and the situation reported at Goose Lake can be more easily understood. The low oxygen consumption of the animals from Goose Lake in the late winter might thus be described as a result of a compensatory adjustment in metabolic economy toward the most critical environmental factor at that time. Such an adjustment, although not large, might be of vital importance to the animals, living as they do through long periods with oxygen supplies which must be approaching the lowest limit that they can tolerate and still carry on an aerobic metabolism.

Experimentally induced adjustment to low oxygen content by exposure to environment with low oxygen has previously been reported in the literature. Lund (1921) reported that repeated exposure of *Planaria* to low oxygen tensions caused less increase in oxygen uptake after each experiment. Hiestand and Singer (1934) also found that leeches responded by "acclimatization" after repeated exposure to water with low oxygen content, and that they gradually went back to their former state after the experiments were terminated. The question whether these experimentally induced changes in oxygen uptake are comparable with the natural reduction occurring in amphipods cannot be answered but the similarity is striking. On the other hand the benefit of such an adjustment for the amphipods seems quite clear and the remarkable ability of the animals to get along with the low amount of oxygen available in the late winter can be more easily understood. Adjustment to a high oxygen consumption in the winter, such as that reported in literature dealing with other poikilotherms, would in the case of the Alaskan amphipods

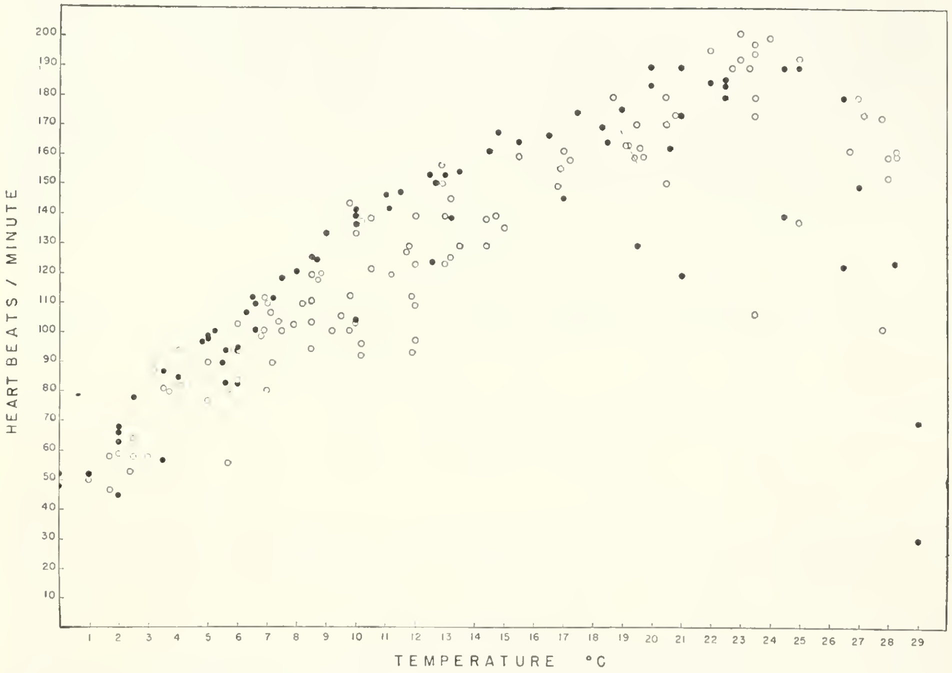

FIGURE 5. Heart rate in beat per minute plotted against time. Open circles represent summer animals and solid dots, winter animals.

be unfavorable as it is of more moment for them to survive the winter than to maintain a high metabolism when the oxygen supply is critically scant.

The Q_{10} as calculated from Figure 3 shows values around 2, the Q_{10} in the winter being slightly lower in value than in the summer (see Table II). Scholander *et al.* (1953) have given a complete discussion of the problem of Q_{10} as an indication of an animal's state of adaptation to the environment. They concluded that physiological adaptation of poikilotherms to cold has its manifestation in the lateral displacement of the metabolism temperature curve but not in the change of its slope as expressed as change in Q_{10}. In the amphipods the summer and winter values for Q_{10} did not differ appreciably.

The changes in the upper lethal temperature levels follow fairly closely those expected on the basis of previous reports upon temperature tolerances of poikilotherms, adding no new information regarding this type of adjustment.

The changes in heart beat, although quite small, show on the other hand some rather interesting trends. The differences between the summer and winter rates above 19° C. indicate that the summer animal can increase its heart rate beyond the point at which the winter animal's heart ceases to function normally. The over-all lower heart rate of the summer animal below 20° C. is not easily understood. Fox (1939) found that the marine polychaete worm *Perinereis cultiifera* from English waters had the same rate of heart beat at 14° C. as the same animal from the Mediterranean had at 20° C. He also found that crustaceans from colder waters around the British Isles had, on the average, a higher rate of heart beat at a given temperature than those from warmer waters of the Mediterranean despite the fact that arctic animals are usually bigger than individuals of the same species from warmer waters, and an opposite relationship between size and heart rate seems to exist commonly among invertebrates. The present observation of changes in heart rate according to season within the same animal population could be compared to the findings reported by Fox (1936) on marine invertebrates taken from waters at different latitude and temperature, except that the changes observed in the Alaskan amphipods might be explained as being brought about through adjustments to the yearly variation in the environment. The particular component or components of the environment which induce this reaction, however, cannot be fully determined. A direct parallel to the cases reported upon by Fox, with an apparent relationship between the physiological responses to laboratory-imposed temperature changes and the temperature of the animal's original environment, would only be possible if the oxygen content of the water were plentiful. Under the reported conditions there is also the probability that the heart rate would increase in the winter so as to compensate for the lower oxygen content of the water at that time of year.

TABLE II

Q_{10} of O_2 consumption in Gammarus limnaeus (Smith)

Time	Temperature °C.		
	0–10	5–15	10–20
Summer	—	2.4	2.0
Winter	2.1	2.1	1.8

This and related studies suggest that considerable physiological flexibility seems to be present in individual aquatic poikilotherms, making it possible for them to make adjustment to comparatively large variations in their environments. The differences between the winter and summer temperature in Alaska equal the difference in the sea around Britain and in the Mediterranan. One population of amphipods encounters the Alaskan seasons by adaptive physiological changes occurring during the life of individuals. Two isolated sections of a population might continue to live safely within the extremes of their physiological adaptability without offering a visible hold for natural selection to differentiate their physiological processes.

It is a great pleasure for me to acknowledge the guidance, interest, and kind criticism by Dr. Laurence Irving during the course of this study. I would also like to thank Miss Mildred Wagner for the excellent help in doing determinations of dissolved O_2, and the drawings; my wife, Hildur Krog, for assistance in the calculation of the data; Dr. P. F. Scholander for kindly reviewing the manuscript and for valuable suggestions concerning the presentation of the results, and Dr. Leslie Hubricht for identifying the amphipods.

SUMMARY

1. A year's cycle of the oxygen consumption of the amphipod, *Gammarus limnaeus* (Smith), has been recorded. The metabolism has been related to the environmental factors encountered in a shallow Alaskan lake, which is ice-covered during seven winter months.

2. A seasonal change in the metabolic rates of the animals accompanied changes in the available oxygen. These resulted in decreased oxygen consumption during the winter when the oxygen supply in the lake was low.

3. The lethal temperature of the amphipods changed from 26° C. in winter to 30°–32 C. in summer.

4. The rate of the heartbeat of the animals varied slightly with the season, with a higher average rate at temperatures below 20° C. in winter than in summer.

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SEROLOGICAL COMPARISONS AMONG FOUR CLASSES OF MOLLUSCA[1]

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Serological comparisons of the proteins of organisms are a means of studying the results of biochemical evolution. With the precipitin test the chemical similarities of the proteins of animals can be measured quantitatively and the degrees of relationship thereby estimated. The purpose of this paper is to report on some serological comparisons of the proteins from the four Classes Amphineura, Gastropoda, Pelecypoda, and Cephalopoda in the Phylum Mollusca.

Materials and Methods

Antigens

Table I lists the species of Mollusca from which antigens were obtained. Sera from *Octopus vulgaris* and *Sepia officinalis* were collected at the Stazione Zoologica di Napoli, Naples, Italy in the summer of 1948. Serum from *Busycon caricum* was obtained at the laboratory of the United States Fish and Wildlife Service, Beaufort, North Carolina, in the summer of 1951. The other antigens were collected at the Friday Harbor Marine Laboratory, University of Washington, Friday Harbor, Washington, in the summer of 1952. 'Merthiolate' in a final concentration of 0.01 per cent (1 : 10,000) was added as a preservative at the time of collection.

Antisera

The antisera used in this study were produced in healthy, adult rabbits. Table I shows the schedule of injections followed for the production of these antisera. Multiple series of injections were given to all rabbits except the one which produced the antiserum against the proteins of *Tonicella lineata*. This rabbit received a single series of injections; seven injections were given on alternate days—1.0, 1.5, 2.0, 2.0, 5.0, 5.0, and 5.0 ml. The fifth and sixth were intraperitoneal and the others were intravenous. Each rabbit was given a trial bleeding from the median artery of the ear eight days after the last injection of a series. At the end of the injection schedule those rabbits with good levels of antibody were completely exsanguinated by intracardial puncture after a fast of 18 hours to obtain sera free from dissolved lipids. Whole blood, obtained either by bleeding from the median artery of the ear, or by intracardial puncture, was placed in lusteroid centrifuge tubes, permitted to clot, rimmed, and held at 0 ± 2° C. for 1 to 10 hours. Expressed serum was

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[2] Public Health Service Predoctorate Research Fellow of the National Microbiological Institute.

TABLE I

Mollusca used in serological comparisons and injection schedules used to produce antisera

Species	Serum protein gms./100 ml.	Injection schedule	Series of injections
PHYLUM MOLLUSCA			
Class Cephalopoda			
Octopus vulgaris Linnaeus	1.75	1.0, 1.5, 2.0 ml., iv*., alt. days†	4
Sepia officinalis Linnaeus	4.20	1.0, 1.5, 2.0 ml., iv., alt. days	4
Class Gastropoda			
Argobuccinum oregonensis (Redfield)	3.10	1.0, 1.0, 2.0, 2.0 ml., iv., alt. days	2
Busycon caricum (Gmelin)	2.40	1.0 1.5 2.0 ml., iv., alt. days	4
Class Pelecypoda			
Macoma nasuta Conrad	1.27	1.0, 1.5, 2.0, 2.0 ml., iv., alt. days	2
Modiolus modiolus Linnaeus	0.27	1.0, 2.0, 2.0, 2.0 ml., iv., alt. days	2
Pecten hericus Gould	0.65	1.0, 1.0, 2.0, 4.0 ml., iv., alt. days	2
Pecten hindsii (Carpenter)	0.45	1.0, 1.0, 2.0, 4.0 ml., iv., alt. days	2
Pododesmus macroschisma Deshayes	0.47	1.0, 2.0, 2.0, 2.0 ml., iv., alt. days	2
Class Amphineura			
Cryptochiton stelleri Middendorf	2.47	2.0, 1.5, 2.0 ml., iv., alt. days	4
Katharina tunicata Wood	4.80	1.0, 1.5, 2.0 ml., iv., alt. days	4
Mopalia muscosa (Gould)	1.07	1.0, 1.5, 2.0 ml., iv., alt. days	4
Tonicella lineata Wood	0.67	1.0, 1.5, 2.0, 2.0 ml., iv., alt. days, 5.0, 5.0 ml., ip**., 5.0 ml., iv., alt. days	1

*iv = intravenously, **ip = intraperitoneally, † alt. days = alternate days

poured into clean, dry lusteroid centrifuge tubes and centrifuged for 30 minutes at 375 g. The resulting clear serum was decanted, sterilized by passage through Seitz filters, put into sterile serum vials and frozen at − 20° C. until used.

METHODS OF TESTING

Quantitative measures of precipitin reactions were made with the Libby (1938) photronreflectometer, using the methods outlined by Boyden and DeFalco (1943) and Leone (1949). Precipitin tests were performed by adding a constant amount of antiserum to doubling dilutions of antigens. The proteins were diluted with 0.9 per cent saline buffered with $M/150$ phosphate salts. 'Merthiolate' in final concentration of 0.01 per cent (1 : 10,000) was added as a bacteriostatic agent to the saline diluent. Initial dilutions of antigens were made on the basis of the known concentration of protein (Table 1); dilutions ranged from 1 : 62.5 to 1 : 4,096,000. Control readings of antigen and saline, and antiserum and saline, were subtracted from the total turbidity readings. Corrected values for the antigen-antibody precipitates were plotted with the turbidities on the ordinate and the antigen dilutions on the abscissa. Representative curves are shown in Figure 1. A value proportional to the area under each curve was obtained by a summation of the turbidity values (Boyden and DeFalco, 1943). Duplicate tests yield "area" values which are within ± 3 per cent of each other. The "areas" of the homologous and heterologous reactions were used to establish the relationships among the various proteins which were compared. To compute the percentage relationship, the summation of

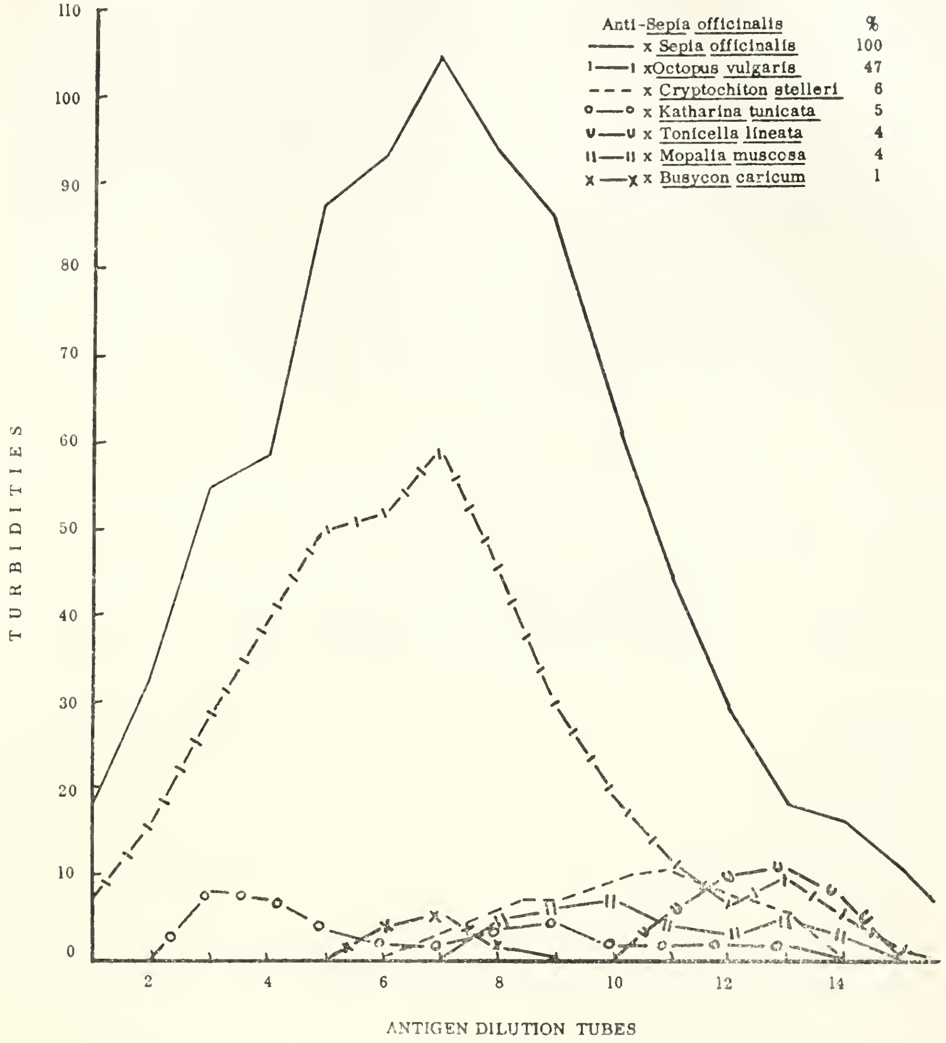

Figure 1. Precipitin reaction curves obtained with the antiserum produced against *Sepia officinalis*. Values proportional to the areas under the curves are obtained by summating the turbidity values. Comparisons are made of the "areas" to determine the per cent relationship among the species represented.

the homologous reaction was divided into the summation of the heterologous reaction and the quotient multiplied by 100.

Experimental Results

All antisera produced using the schedules of injections given in Table I had high levels of antibodies. Table II gives a summary of the serological relationships which were calculated from the precipitin tests performed with the various antigens and antisera. The percentage values in Table II are based on averages of tests done in duplicate.

The antiserum produced against the proteins of *Octopus vulgaris* had the strongest cross-reaction with the proteins of the other cephalopod, *Sepia officinalis.* Proteins from the gastropod *Busycon caricum* were slightly more reactive with this antiserum than were the proteins from the several amphineurans which were tested. The proteins from the pelecypods *Pecten hericus* and *P. hindsii* had no measurable reactions with this antiserum.

The antiserum produced against the proteins of *Sepia officinalis* had the strongest cross-reaction with the proteins of *Octopus vulgaris.* This antiserum did not permit us to distinguish effectively between the gastropods and amphineurans, the proteins of both of which were measurably reactive. The pelecypods *Pecten hericus* and *P. hindsii* had no measurable reactions with the antiserum.

TABLE II*

Per cent relationships among species from four classes of Mollusca

Antisera / Antigens	*Octopus vulgaris* Cephalopoda	*Sepia officinalis* Cephalopoda	*Argobuccinum oregonensis* Gastropoda		*Busycon caricum* Gastropoda	*Macoma nasuta* Gastropoda	*Modiolus modiolus* Pelecypoda	*Pecten hericus* Pelecypoda	*Pecten hindsii* Pelecypoda	*Pododesmus macroschisma* Pelecypoda	*Cryptochiton stelleri* Amphineura		*Katharina tunicata* Amphineura	*Mopalia muscosa* Amphineura	*Tonicella lineata* Amphineura
Octopus vulgaris	100	47	0	5	1	0	0	0	0	0	1	1	1	1	0
Sepia officinalis	69	100	0	3	0				0		2		8	0	0
Argobuccinum oregonensis		0	100	100	33			0	0		0		0		0
Busycon caricum	21	1	58	34	100	0	0	0	0		7	0	2	0	0
Macoma nasuta						100	0			0					
Modiolus modiolus				2		0	100			13		0	0		0
Pecten hericus	0	0	0	3	0	22	4	100	56	0	0	0		0	0
Pecten hindsii	0	0	0		0			74	100			0	0	0	
Pododesmus macroschisma						0	5			100					
Cryptochiton stelleri	16	6	0	0	2			19	6		100	100	79	65	31
Katharina tunicata	14	5	0	1	12	0	0	19	0	0	54	80	100	69	25
Mopalia muscosa	13	4	0								36	65	63	100	26
Tonicella lineata		4	0		0			0			10	20	24	23	100

* Comparative values are in the vertical columns. Experimental error: ± 3 per cent relationship. The columns headed *Argobuccinum* and *Cryptochiton* contain sets of data for two antisera.

The two antisera which were produced against the proteins of the gastropod *Argobuccinum oregonensis* were specific with respect to cross-reactivity with the proteins from representatives of the other Classes in the Phylum Mollusca. Both antisera had strong cross-reactions with the other gastropod, *Busycon caricum.* The less specific of the two antisera was slightly reactive with the proteins from the cephalopods *Octopus* and *Sepia,* the pelecypods *Modiolus* and *Pecten* and the amphineuran *Katharina.* The more specific of the two anti-*Argobuccinum* sera gave measurable cross-reactions only with the proteins of the other gastropod *Busycon.*

The proteins of the other gastropod, *Argobuccinum,* were most reactive with the antiserum to *Busycon caricum.* The reactivity of the proteins of the amphineuran *Katharina tunicata* with this antiserum was strong as compared to the slightly positive results, and negative results obtained with the proteins of the other amphineurans. Of the cephalopods, only the proteins of *Octopus* were reactive and to a small extent. Proteins from the pelecypod *Pecten* had no measurable reactivity with the anti-*Busycon* serum.

The antiserum to the proteins of the pelecypod *Macoma nasuta* was strongly reactive with the proteins of *Pecten hericus,* but gave negative results with the proteins of the other pelecypods *Modiolus* and *Pododesmus.* Negative also were the results of tests performed with representatives from the other Classes in the Phylum.

The anti-*Modiolus* serum was slightly, and equally, cross-reactive with the proteins of the pelecypods *Pododesmus* and *Pecten.* Proteins from the pelecypod *Macoma* were not measurably reactive. Proteins from representatives from the Gastropoda, Cephalopoda, and Amphineura were likewise not measurably reactive.

Proteins from *Pecten hericus* and from *Pecten hindsii* each induced the formation of strikingly similar antisera. Good differentiation between species was obtained with both antisera when they were tested with the proteins of the two pectens. Cross-reactions were obtained between the proteins of the amphineurans *Cryptochiton* and *Katharina* and anti-*Pecten hericus,* and between *Cryptochiton* and anti-*Pecten hindsii.* Proteins of other amphineurans and proteins from the cephalopods and gastropods gave negative results when tested with these antisera.

The antiserum produced against the proteins of the pelecypod *Pododesmus macroschisma* was cross-reactive with the proteins of the pelecypod *Modiolus modiolus.* Results obtained with proteins from tests performed between this antiserum and the proteins from species representative of the other Classes of the Phylum Mollusca were negative.

The two antisera which were produced against the hemocyanins of the amphineuran *Cryptochiton stelleri* had differing specificities. Within the Amphineura, however, the sequence of relationship for cross-reacting proteins was the same for each antiserum. Proteins from *Katharina tunicata* had the strongest cross-reaction, followed by *Mopalia muscosa* and then *Tonicella lineata.* With one of the antisera, the proteins of the gastropod *Busycon* were more reactive than were the proteins of the cephalopods *Sepia* and *Octopus*; however, with this same antiserum, the proteins of the other gastropod, *Argobuccinum,* were negative. The other antiserum against *Cryptochiton* had a slight cross-reaction with the proteins of *Octopus* and no measurable reaction with the proteins of *Busycon.* The latter antiserum was also not measurably reactive with the proteins of the pelecypods.

The antiserum produced against the proteins of *Katharina tunicata* had its strongest cross-reaction with the proteins of *Cryptochiton. Mopalia* and *Tonicella* were next most reactive in that order. Of the species from other Classes the proteins of the cephalopod *Sepia officinalis* were most reactive. The proteins of the cephalopod *Octopus* were reactive to a lesser extent, as were the proteins of the gastropod *Busycon.* The proteins of the pelecypods had no detectable reactions with this antiserum.

Proteins from *Katharina tunicata* and *Cryptochiton stelleri* were approximately equally reactive with the anti-*Mopalia* sera and more so than were the proteins of *Tonicella lineata.* The proteins of *Octopus vulgaris* were slightly cross-reactive with this antiserum.

Reactions involving the antiserum produced against the proteins of *Tonicella lineata* were restricted to the proteins of the amphineurans. By means of the antiserum we were unable to distinguish effectively among the proteins of the species *Cryptochiton stelleri, Katharina tunicata,* and *Mopalia muscosa.*

DISCUSSION

Only a few reports in the literature provide serological data on interclass relationships among the mollusks.

Galli-Valerio (1916) produced antisera against tissue-extracts of the pelecypod *Anodonta anatina* (Linnaeus). His only heterologous precipitin tests were with similar extracts from the gastropod *Limnaea stagnalis* (Linnaeus), which produced precipitates.

Erhardt (1931) produced antisera against the gastropods *Arion empericorum* Ferussac and *Limnaea stagnalis* (Linnaeus). The stronger anti-*Arion* serum cross-reacted well with the proteins of the other gastropods, but gave negative results in tests with the proteins of the amphineuran *Chiton marginatus* Pennant.

Makino (1934) employed precipitin, complement fixation and anaphylactic reactions with saline-extracted antigens from the pelecypods *Arca inflata* Reeve, *Cytherea meretrix* Linnaeus, *Ostrea gigas* Thunberg, *Paphia philippinarum* Adams and Reeve, the gastropods *Haliotis gigantea* Chemnitz, *Rapana thomasi* Crosse, *Turbo coronatus* (Gmelin) and the cephalopods *Sepiella japonica* Sasaki, and *Polypus* [= *Octopus*] *variabilis* Sasaki. Within each Class the cross-reactions between antigens and antibodies were strong. Cross-reactions between reagents which were representative of different Classes of mollusks were weak or doubtful, or gave negative results. His precipitin data were extensive and he judged that they indicated the gastropods resembled the pelecypods more than they did the cephalopods. This judgment was based on "ring test" data, the differential titers of which were within the experimental error of method, and generally on "plus" versus "plus-minus" reactions. In our opinion his data revealed no conclusive order of relationship among the three groups of mollusks.

Kuramoto (1933) made extensive interclass serological comparisons of representative species of pelecypods, gastropods and cephalopods. He produced antisera against the pelecypods *Ostrea gigas* Thunberg, *Corbicula japonica* Prime, *Cristaria plicata* Leach, *Cytherea meretrix* Linnaeus and *Tapes philippinarum* Adams and Reeve, the gastropods *Haliotis gigantea* Chemnitz, and *Viviparus japonicus* Martens and the cephalopods *Euprymna morsei* Verrill, and *Octopus membranaceus* Quoy. His precipitin ("ring-tests") and complement fixation data show, without question, a closer relationship between the gastropods and cephalopods than between either of these and the pelecypods. This work is singularly exceptional, in comparison to the findings of others, in that his results give strong interclass serological reactions which reciprocally verify one another.

Chestnut (1943) employed the turbidimetric analysis of precipitin reactions in his study of molluscan relationships. He produced antisera against the tissue-proteins of the pelecypods *Ostrea gigas* Thunberg and *Ostrea virginica* (Gmelin), the hemocyanins of the gastropods *Fasciolaria gigantia* Kiener, *Busycon perversum* Linnaeus, *Busycon caricum* (Gmelin), and *Aplysia protea* (Rang) and a cephalopod *Octopus* sp. The antisera against the pelecypod proteins were Class-specific, as were all but one of the antigastropod sera. A slight cross-reaction was obtained between the anti-*Aplysia* serum and the proteins of the pelecypods. Other interclass cross-reactions were obtained with the anti-Octopus serum and the proteins from both the pelecypods and the gastropods. The gastropods were indicated to be slightly more closely related to the cephalopod than were the pelecypods.

Wilhelmi (1944) obtained reciprocal cross-reactions between antigens and antisera of the gastropod *Busycon caricum* (Gmelin) and the pelecypod *Pecten irradians* Lamarck.

Our serological data were inconclusive with respect to the establishment of a definite sequence of relationship among the four Classes of the Phylum Mollusca. The values for interclass relationships were generally low and approximately at the limit of the sensitivity of the serological methods which were employed. Multiple series of injections were given to induce the formation of powerful antisera. It was seemingly beyond the capabilities of the rabbits to produce antisera by means of which we could discriminate effectively among the cross-reacting proteins from the different Classes. Most of the proteins from species which were in the same Class as the homologous species were reactive with a given antiserum and readily distinguished from proteins from another Class. Were it not for the fact that the interclass data were chiefly at the limit of the ranges of the antisera, and close to the experimental error in the method, the negative results might otherwise have been significant as a means of distinguishing, in part, among the Classes. An inspection of the interclass data reveals several instances where the proteins of one species within a Class gave a measurable cross-reaction with an antiserum while the proteins of a second species from the same Class gave a negative result.

The pelecypod proteins gave fewer positive interclass reactions than did the proteins of the representatives of the other Classes. Moreover, most of the antisera produced against the proteins of the pelecypod species were more specific than the antisera produced against the proteins of species from other Classes. This pattern of cross-reactions for the pelecypods might have been due, in part, to the noncorrespondence of their proteins with the proteins of the species from the other Classes. The proteins of the pelecypods were in extracts obtained from homogenates of the whole bodies of organisms. Proteins from species in other Classes were the hemocyanins of the sera. The low cross-reactivity of the proteins of the amphineuran *Tonicella lineata,* which were obtained also as extracts and leachings from the bodies of the organisms, likewise might have been due to their lack of correspondence with the serum proteins of the other amphineurans. More difficult to comprehend are differences in cross-reactivity, in view of the generally aspecific nature of interclass serological reactions, of the hemocyanins from species in one Class with the antihemocyanin sera representative of another Class. The hemocyanins of the gastropod *Argobuccinum,* for example, failed to give a measurable cross-reaction with the anti-*Cryptochiton* serum, while the hemocyanins of the gastropod *Busycon* had seven per cent of measurable correspondence with the homologous reaction. The difference between the reactivities of *Argobuccinum* and *Busycon* exceeds the limits of the experimental error of the methods. Both of the antigens were collected and preserved in the same manner. Their places of origin were greatly different, but this fact should not be of importance if the same laws of inheritance and principles of systematics and evolution apply to the conservatively inherited proteins of the serum as apply to other essential characters of these organisms. Lipoprotein-anti-lipoprotein reactions have been known (Boyden, 1936; Cumley, 1939; Wilhelmi, 1944) to account for otherwise unpredictable serological reactivity involving hemocyanins and other proteins from invertebrates. Lipoidal substances were not removed from the serum antigens which were used in our experiments. The presence of lipid materials in the serum of invertebrates might be a superficial trait, a consequence of the dietary habits of the animals, hence, under environmental control, and of trivial systematic importance in serological comparisons. Lipid materials bound to proteins as a consequence of genetically controlled syntheses are of unquestioned value in serological comparisons. The relatively aspecific nature of the fatty component of lipoproteins would provide the greatest usefulness in studies of serological correspondence among distantly related groups of animals. It was this reasoning which led us to use antigens which had not been defatted.

A critical evaluation of the interclass serological data in this paper, and in earlier reports (Erhardt, 1931; Kuramoto, 1933; Makino, 1934; Chestnut, 1943; and Wilhelmi, 1944), does not support Knight's suggestion (1952, p. 44) to merge the isopleuran (amphineuran) and anisopleuran (gastropod) mollusks into a single Class, the Gastropoda. By assuming the generally lowered interclass reactivities of the pelecypods (Table II) to be due to the lack of correspondence of their tissue-extract proteins with the hemocyanins of the sera of other groups and by recalling that most of the measurable interclass reactivities are within or near the experimental error of the method, all of the groups would stand distantly related, and approximately equally so. In this interpretation the four Classes Amphineura, Gastropoda, Pelecypoda, and Cephalopoda would be retained as commonly accepted.

Cross-reactions between serological reagents involving proteins from pelecypods, gastropods and cephalopods have been reported by Kuramoto (1933), Makino (1934), and Chestnut (1943). Each of these workers obtained results which indicated that the gastropods and cephalopods are more closely related to each other than either of them is to the pelecypods. Their data indicate, also, a closer relationship between pelecypods and gastropods, than between pelecypods and cephalopods. Our data yielded no conclusive information on the relative amounts of serological correspondence among the three groups.

Both of the anti-*Pecten* sera had reactions with the proteins of the amphineurans which were inconsistent with the other serological data in Table II. We can offer no explanation for the reactions. Subsequently-produced anti-*Pecten* sera gave no such cross-reactions with the proteins of the amphineurans. The reactions of the anti-*Macoma* serum are also unusual because of the strong cross-reaction of this serum with *Pecten* and the failure of the serum to react with the proteins of other pelecypods. These "anomalous" data are included in our results to illustrate the occasional occurrence of unverifiable serological reactions which are in all probability due to unusual responses of rabbits to the antigens being injected.

SUMMARY

1. Serological comparisons were made with precipitins of proteins from molluscan species representative of the four Classes Amphineura, Gastropoda, Cephalopoda and Pelecypoda.

2. Interclass reactions were approximately at the limit of the sensitivity of the turbidimetric method which was employed.

3. Knight's proposal to include the amphineurans in the Class Gastropoda is not supported by the serological data. The analysis and integration of the serological data already in the literature, with the data in this work, provide additional evidence that the four groups should be retained as distinct Classes.

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STUDIES ON THE SYMBIOTIC YEASTS OF TWO INSECT SPECIES, LASIODERMA SERRICORNE F. AND STEGOBIUM PANICEUM L.[1]

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The organs and tissue cells of many insects harbor living microorganisms which seem to exert no harmful effect on their hosts. In fact, some of them have been found to bear a symbiotic relationship with the insects. It has been shown in many instances, especially in termites, blood-sucking insects and two anobiid beetles, *Lasioderma serricorne* F. and *Stegobium* (*Sitodrepa*) *paniceum* L., that symbionts can play an important part in the nutrition of their host (Fraenkel, 1952; Buchner, 1953).

The mycetom which houses the yeast-like symbionts of *Stegobium* was first described by Karawaiew (1899), and the true nature of the symbionts first recognized by Escherisch (1900). Later Buchner (1912), Heitz (1927), Breitsprecher (1928), Koch (1933), Fraenkel and Blewett (1943), Blewett and Fraenkel (1944) and Pant and Fraenkel (1950) added to our knowledge of the relationship of the symbionts to their insect hosts in *Lasioderma* and *Stegobium*. Koch (1933) recognized that the symbionts of *Stegobium* exerted a function in the nutrition of the host which was similar to that of yeast. Blewett and Fraenkel (1944) showed that the symbionts were sources of many of the vitamins of the B-complex for their hosts. This present study continues and extends the previous work by Blewett and Fraenkel and is also concerned with the culture of the yeasts outside the body and the effect of transplanting them into the foreign host. Some of the results have already been briefly reported (Pant and Fraenkel, 1950).

Materials and Methods

Cultures of *Lasioderma serricorne* and *Stegobium paniceum* were maintained on wholemeal flour and wheat bran with the addition of 5% dried debittered brewers yeast. The cultures had to be covered with tightly fitting wire gauze tops because of the tendency of the adult beetles to cut through muslin covers. In order to obtain a large number of eggs, adult beetles were maintained on a small quantity of white flour plus 5% yeast. Eggs laid in this medium were recovered by sifting through a 60-mesh sieve.

The basic diet was the same as that described previously for *Tenebrio molitor* (Fraenkel *et al.*, 1950) and consisted of 20 parts casein, 80 parts glucose, 1 part cholesterol and 2 parts McCollum's salt mixture no. 185. To this mixture the following vitamins of the B-complex were added (expressed as μg. per gram of the dry diet): thiamin 25, riboflavin 12.5, nicotinic acid 25, pyridoxin 12.5, pantothenic acid 25, choline chloride 500, inositol 250, folic acid 2.5 and biotin 0.1. In some cases the water-insoluble residue of yeast was used in the place of biotin (Fraenkel and Blewett, 1943).

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The tests were carried out in 2 × 1 inch shell vials closed by one-holed corks with muslin tops. Ten newly hatched larvae were added to each tube and each test was performed in duplicate. Each tube contained two grams of food. All tests were carried out in a constant-temperature-humidity chamber of 27° C. and 70% relative humidity.

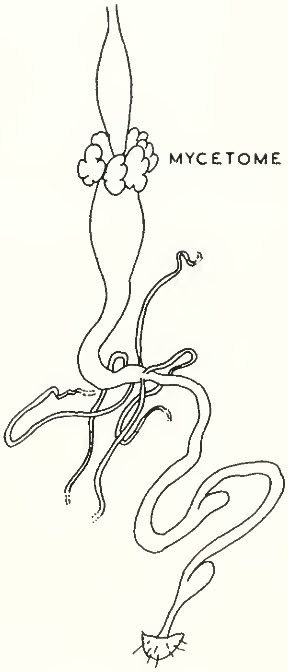


FIGURE 1. Intestine of the adult *Lasioderma serricorne,* showing the mycetoms at the junction of fore- and midgut.

For the study of the symbionts *in situ,* sections were cut and stained with Delafield's hematoxylin and eosin or orange G. Smears of mycetomic tissue were stained with Wright's stain or were smeared on to the slide with a drop of India ink. The latter method proved very suitable for the study of the shape of the cells.

For the graphic representation of results, the total number of adults formed was plotted against time. Both *Lasioderma* and *Stegobium* spin a cocoon previous to pupation. Most of these cocoons are fixed on to the glass wall of the vials leaving open a window to the inside of the cocoon through which the insects can be observed. Thus the time of pupation and emergence of the adults can be accurately determined by frequent inspections.

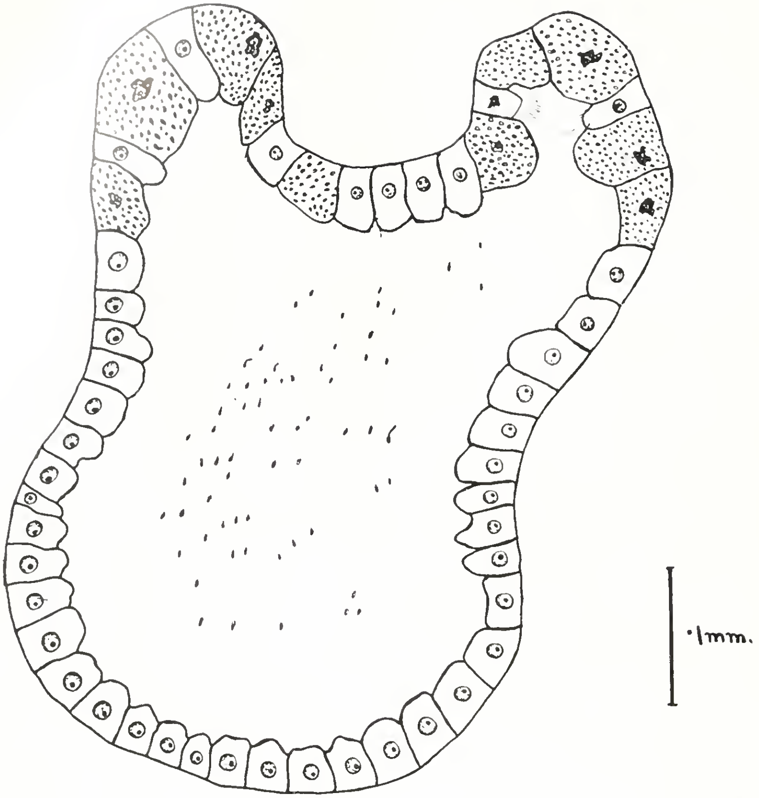


FIGURE 2. Transverse section through two lobes of the mycetom of the *Stegobium paniceum* larva, showing location of symbionts in the mycetom and lumen of the intestine.

OBSERVATIONS AND EXPERIMENTS

1. *Mycetoms and symbionts*

The mycetoms of *Lasioderma serricorne* are similar to those of *Stegobium paniceum* which have already been described several times (Karawaiew, 1899; Buchner, 1912; Heitz, 1927; Breitsprecher, 1928). However in *Lasioderma* they consist of 6 lobes or protrusions situated at the junction of fore and midgut which are evaginations of the wall of the midgut, and which are in continuation with the lumen of the gut (Fig. 1). As in *Stegobium,* they consist of large cells containing symbionts and irregular shaped nuclei. The other cells of the epithelium are without microorganisms and have round nuclei and fringed borders (Fig. 2).

In newly hatched larvae the mycetoms are not externally differentiated. It is after a period of 5–7 days that the mycetoms acquire their characteristic lobed shape. In the prepupae the mycetomes become reduced in size and are no longer prominent. They are further reduced in the pupae, but are fully developed in the adults, where each lobe is outwardly subdivided into three sub-lobes. In *Stegobium,* which has a four-lobed mycetom, the shape of the organ changes markedly in the adult, by the development of six tubular appendages which arise from each lobe.

The symbionts of *Stegobium* were first identified by Escherisch (1900) as organisms which look like yeasts and multiply by budding. They differ markedly in size and shape in the two species (Figs. 3 and 4). In *Lasioderma* the cells are broadly oval, 2 to 3.5 μ wide and 2 to 4.5 μ long, with only one bud attached to a cell, except for the pupa, where two buds per cell are common. The buds are apical and the point of attachment is broad. No mycelium or spores were ever observed. In the adults the symbionts are slightly smaller and budding is less common. In *Stegobium* the yeasts are elongate, pear-shaped, and pointed at one end to which a single bud may be attached. The point of attachment is very narrow. The size varies from 1.5 to 3.5 μ width and 3 to 6 μ length. These differences in size, shape and budding are so characteristic that the two types can be readily discerned.

2. *Cultivation of the symbionts*

Escherisch (1900) claimed to have cultivated *Stegobium* but Heitz (1927) and Breitsprecher (1928) failed in subsequent attempts. We have successfully cultivated the yeasts of both species in Hansen's solution (peptone 1.0 gm., glucose 5 gm., potassium dihydrogen phosphate 0.3 gm., magnesium sulfate 0.3 gm., and water 100 ml.). The larvae were sterilized by submerging them in 5% chloramine in 70% alcohol for two minutes. They were then washed with sterilized water, and the mycetom dissected out under sterile conditions and transferred into the culture medium. In the case of *Lasioderma,* inoculated medium was kept at 29° C. for 15 to 20 days after which it turned turbid, with the cells settling down

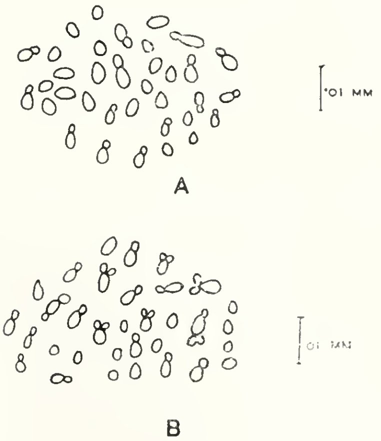


FIG. 3

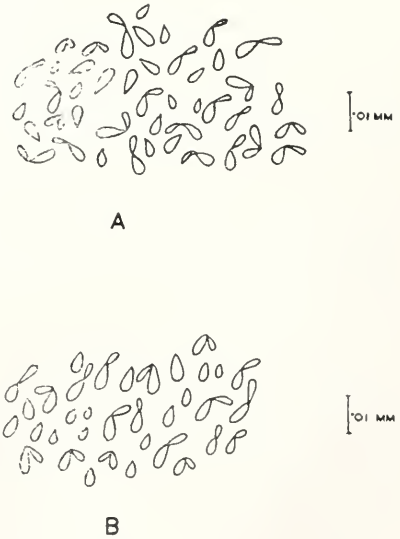


FIG 4

FIGURE 3. The yeast-like symbionts of *Lasioderma serricorne.* A. In the larva. B. In the pupa.

FIGURE 4. The yeast-like symbionts of *Stegobium paniceum.* A. In the larva. B. Cultivated symbionts.

at the bottom. The suspension was then inoculated on to an agar medium where colonies were formed (Fig. 5). The incubation period for the yeasts from *Stegobium* was longer, up to 35 days, and growth was poor compared with that of *Lasioderma* yeasts. No colonies were obtained on the agar medium.

The cultivated yeasts showed the same morphological characteristics as those *in situ* (Figs. 3 and 4). But in very old cultures chains of yeast sometimes developed. *Lasioderma* symbionts could be grown in such profusion that it was possible to conduct feeding trials with them. For inoculation of media mycetoms of larvae, pupae and adults were used with success. Eggs were also employed but given up, owing to the fact that the outer surface, which contained the yeasts, could not be sterilized.

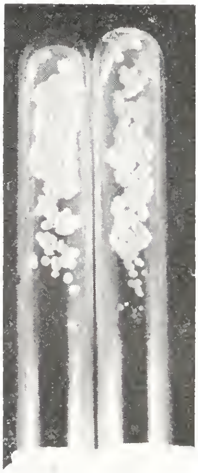


FIGURE 5. A. Cultures of the symbionts of *Lasioderma* on an agar medium. B. Culture of the same symbionts after implantation into *Stegobium* larvae.

The yeasts from either species have never been accurately identified taxonomically. They were provisionally placed by Buchner (1930) under the genus *Saccharomyces* and named *S. anobii*. However they differ from *S. cerevicae* in that they cannot ferment glucose with development of carbon dioxide.

3. *Elimination of yeasts from the insects, and reimplantation*

The insects were deprived of their yeast by a method first described by Koch (1933) and subsequently employed by Blewett and Fraenkel (1944). Eggs were treated with 5% chloramine in 70% alcohol for one minute at room temperature. The yeasts, which are normally transmitted by smearing on the outer surface of the egg, while it passes through the common oviduct, are hereby killed. The eggs are subsequently washed in distilled water. The larvae which hatched from these eggs did not contain symbionts, although the mycetoms developed normally. The offspring from eggs treated in this way will never acquire symbionts, unless reinfected.

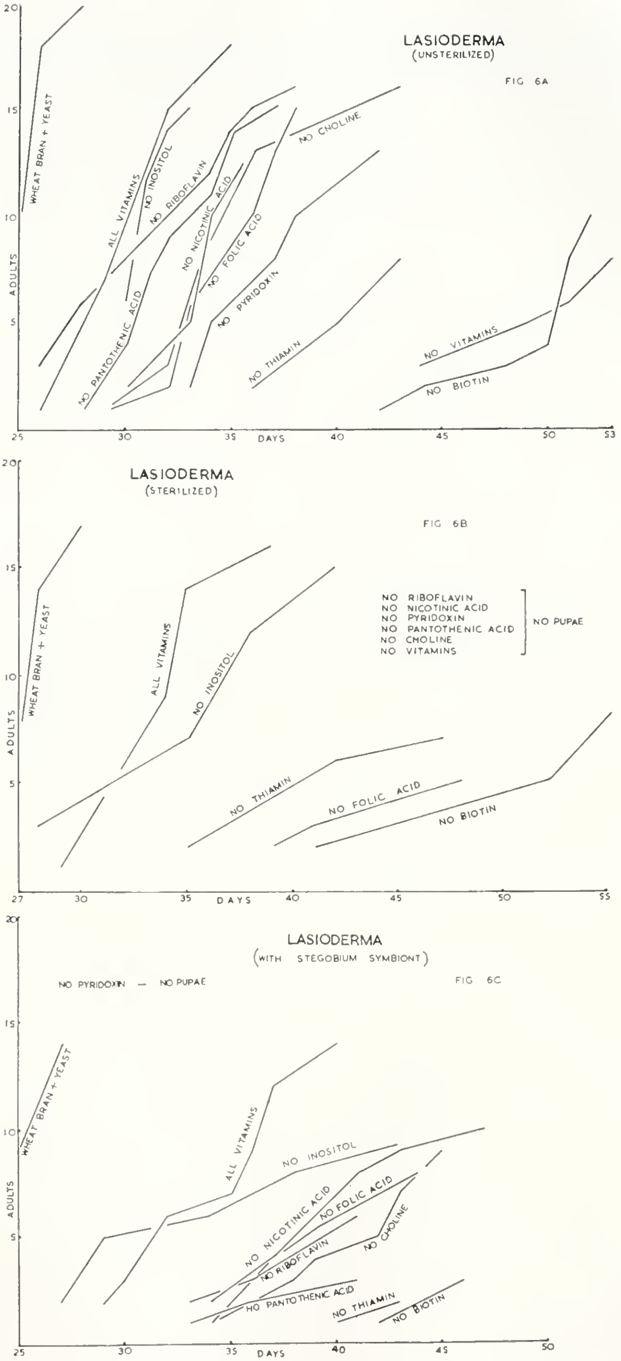


FIGURE 6. Growth of the larvae of *Lasioderma serricorne* on artificial diets in the presence and absence of certain vitamins of the B-complex. A. Normal larvae. B. Larvae without symbionts. C. Larvae which contain the symbionts of *Stegobium*.

When sterile eggs were treated with a suspension of cultivated yeasts the ensuing larvae became reinfected with symbionts. "Sterilized" larvae of *Lasioderma* or *Stegobium* could be reinfected with symbionts by adding to the flour, which was otherwise sterile, one of the following:

1. Culture of cultivated yeast (done only with *Lasioderma* yeasts);
2. 0.1 g. faeces of normal insects in 2 g. of flour;
3. flour from a culture of normal insects.

Sterilized larvae of *Lasioderma* on a diet containing cultivated *Lasioderma* yeast readily became infected. Food which contained faeces of normal larvae did not affect *all* sterilized larvae, but, after two months, *some* individuals of *Lasioderma* and *Stegobium* were found to contain symbionts in their mycetoms. Flour on which normal insects had been feeding previously had no effect on the sterility of the mycetoms of either *Lasioderma* or *Stegobium*. This can be explained by the much smaller amount of faeces present in this sample than the one where faeces had been added.

It was, therefore, concluded that larvae are readily infested by way of mouth, by admixing a culture of the symbiotic yeasts to the food. This method could only be used with *Lasioderma* and not with *Stegobium* whose symbionts did not grow sufficiently well in culture. Sterilized *Stegobium* larvae could, however, be readily infested with the cultivated yeast of *Lasioderma*. The yeasts in the mycetoms retained the characteristic shape of *Lasioderma* symbionts. Cultures were successfully obtained from *Lasioderma* yeasts grown in *Stegobium* mycetoms under circumstances which failed to produce *Stegobium* symbionts. The adults of *Stegobium* with *Lasioderma* yeast produced a generation which contained the typical *Lasioderma* yeast, and this could be observed through several generations.

Stegobium symbionts could be "inoculated" into *Lasioderma* by smearing cultures of them on to sterilized *Lasioderma* eggs. The mycetoms became filled with yeast of the typical shape of *Stegobium* symbionts. The mycetomic tissues of such larvae of *Lasioderma* were inoculated into Hansen's medium and the growth was found to be poor, analogous to the growth of the normal *Stegobium* symbionts.

4. Physiology of Lasioderma and Stegobium symbionts in their normal host

Larvae of *Lasioderma* were grown in the presence and absence of the normal symbionts on artificial diets which contained either the full vitamin complement, or were lacking in one of the vitamins. The tests were performed in two series, one in which the diet contained the water-insoluble fraction of yeast (which contains biotin), and one in which this was replaced by biotin. The results of the two tests were essentially identical. The normal larvae grew relatively well in diets which did not contain riboflavin, inositol, choline chloride, nicotinic acid, pantothenic acid or folic acid. Diets without pyridoxin or thiamin were slightly inferior although the majority of the larvae reached the adult stage. In the absence of insoluble yeast or biotin, growth was very slow and the mortality high. The sterilized larvae grew well in the absence of inositol, very poorly in the absence of thiamin, folic acid or biotin (or insoluble yeast) and entirely failed to grow in the absence of riboflavin, nicotinic acid, pyridoxin, pantothenic acid and choline chloride (Fig. 6).

When sterilized larvae had been reinfested with their own yeast by feeding them on yeast cultures, they grew about as well in the absence of single vitamins as did normal larvae. This clearly indicates that growing the yeast cells in pure culture did not change them physiologically.

A comparison was also made between growth of *Stegobium* in the presence and absence of the symbionts on diets which contained either the full vitamin complement, or had a single vitamin omitted. With the symbionts present the larvae grew very well in the absence of inositol; omission of pyridoxin, riboflavin, nicotinic acid, folic acid or pantothenic acid delayed growth very slightly, but little or no growth took place in the absence of biotin or thiamin respectively. With the symbionts absent, no growth occurred in the absence of thiamin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid, biotin or folic acid. Growth was markedly delayed in the absence of choline chloride and unaffected in the absence of inositol (Fig. 7).

In general, the effect of symbionts in diets lacking in certain vitamins was similar in *Lasioderma* and *Stegobium*. Certain differences which emerged from these

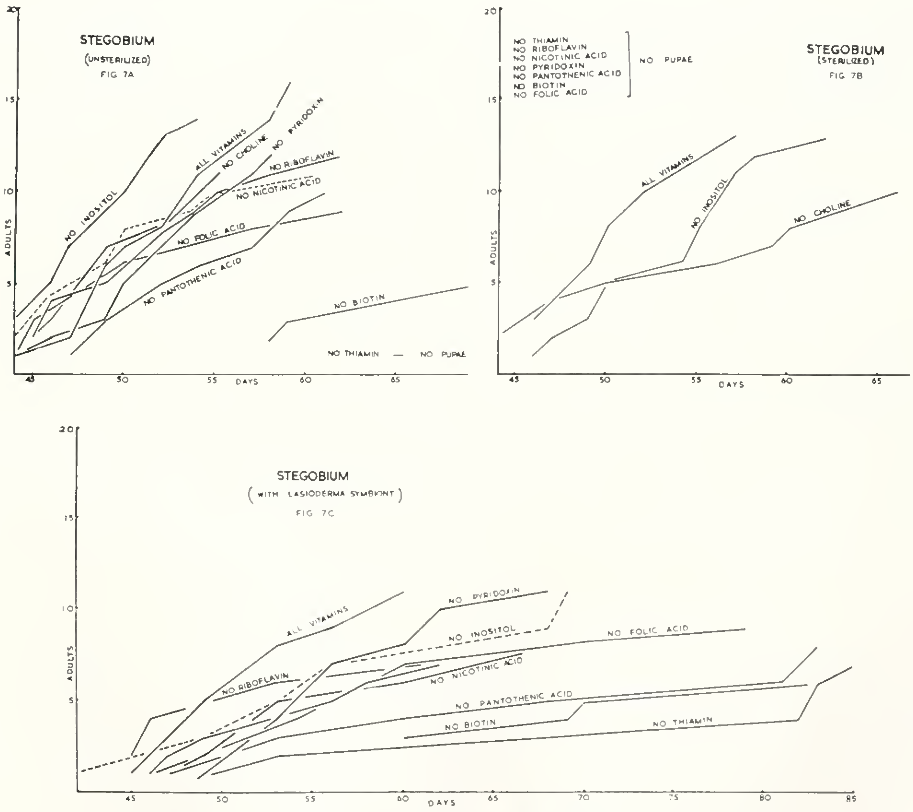


FIGURE 7. Growth of the larvae of *Stegobium paniceum* in the presence and absence of certain vitamins of the B-complex. A. Normal larvae. B. Larvae without symbionts. C. Larvae which contain the symbionts of *Lasioderma*.

tests appear to be connected rather with the requirements of the insects than the properties of symbionts. The normal *Lasioderma* grew slowly in the absence of thiamin, but so also did the larva after removal of the symbionts. Neither the normal nor sterilized *Stegobium* grew in the absence of thiamin. It seems, therefore, that neither *Lasioderma* nor *Stegobium* symbionts supply sufficient quantities of thiamin. The sterilized *Lasioderma* failed to grow in the absence of choline chloride, while choline was not required by the sterilized *Stegobium*.

5. *The effect of the symbionts of Lasioderma and Stegobium in the abnormal host*

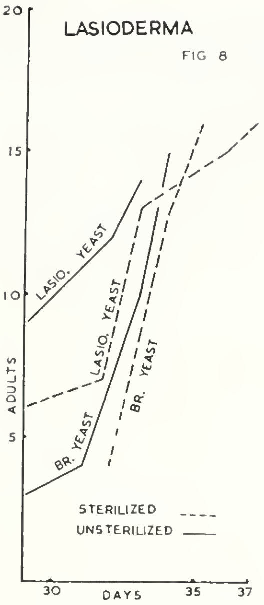
Sterilized *Lasioderma* was infected with the symbionts of *Stegobium* by the method of smearing a culture of *Stegobium* yeasts on the sterilized eggs. The resulting larvae were then submitted to the series of diets, which were lacking in certain factors, as has been described before. Growth of these larvae was better than that of sterilized larvae but inferior to that of normal larvae (Fig. 6). In most instances growth was delayed and the mortality increased. However, in only one case, in the absence of pyridoxin, did the *Lasioderma* larvae with *Stegobium* symbionts entirely fail to develop. In the converse experiment cultivated yeast of *Lasioderma* was mixed with the food on which sterilized *Stegobium* larvae were maintained. The offspring of these larvae were submitted to the tests described above. *Stegobium* larvae with *Lasioderma* yeasts grew about as well as in the presence of their own yeasts in the case of most individual vitamin deficiencies, and even better in the absence of thiamin (Fig. 7).

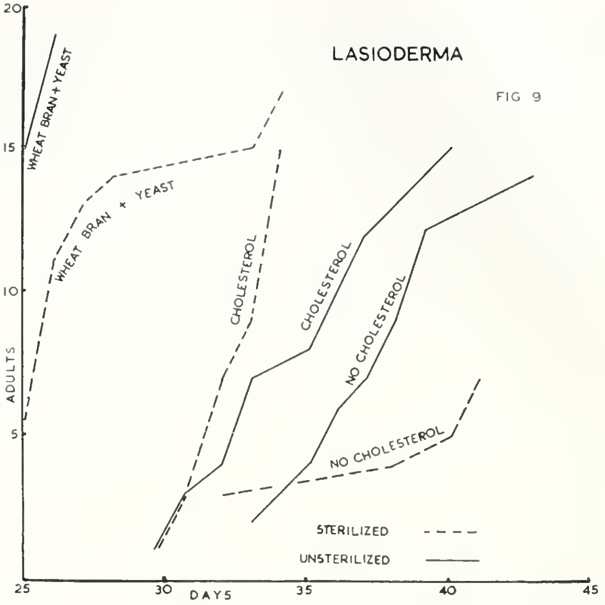
6. *Comparison between the effect of dried brewers yeast and that of cultivated Lasioderma symbionts*

Lasioderma yeasts were cultivated on an agar medium and the yeast cells then carefully separated from the medium and dried; 2.5% of dried symbiotic yeasts were then added to the basic diet, which did not contain vitamins, and their effect compared with that of 2.5% of dried brewers yeast. Both normal and sterilized larvae grew equally well on either diet (Fig. 8). This result was in agreement with the tests previously reported. *Lasioderma,* owing to its symbiotic relationship with yeast, does not need vitamins like other insects. The reason why the normal *Lasioderma* did not grow optimally in the absence of pyridoxin or thiamin, while sterilized *Lasioderma* did so in the presence of *Lasioderma* yeast in the diet, can be attributed to the far larger amounts of symbiotic yeast present in the latter case.

7. *The symbionts as a source of sterols*

By analogy with other insects, *Lasioderma* and *Stegobium* were expected to require a dietary source of a sterol and it was of interest to find out whether the symbionts, in addition to vitamins of the B-complex, also supplied sterols. When cholesterol was omitted from the basic diet of *Lasioderma,* growth of normal larvae was not affected. Sterilized larvae, on the other hand, grew slowly and with high mortality in the absence of cholesterol. When cholesterol was added the efficiency of the diet was at once restored (Fig. 9). It is evident that a sterol deficiency does not arise as long as symbionts are present in the insect. In another test the





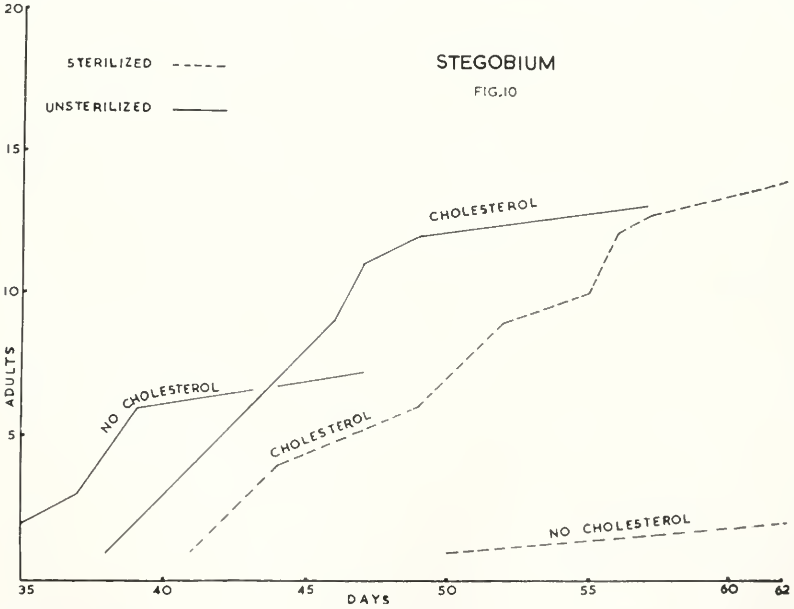


FIGURE 8. Growth of normal and symbiont-free larvae of *Lasioderma* in the presence of dried symbionts or dried yeast as sources of vitamins. L.Y. = Dried *Lasioderma* yeast. Br. Yeast = Dried brewers yeast.

FIGURE 9. Growth of normal and symbiont-free larvae of *Lasioderma* in the presence and absence of cholesterol.

FIGURE 10. Growth of normal and symbiont-free larvae of *Stegobium* in the presence and absence of cholesterol.

presence of the insoluble yeast fraction in the diet resulted in normal growth in normal and sterilized larvae alike, in the absence of cholesterol. This can be attributed to the presence of sterols, largely ergosterol, in the yeast fraction.

Results regarding sterol requirements, corresponding to those reported for *Lasioderma*, were also obtained with *Stegobium* (Fig. 10). Both normal and sterilized larvae grew about equally well in diets which contained cholesterol. Without cholesterol there was a marked difference in both types of larvae. Normal larvae grew rapidly, though there was a high mortality, but there was little growth in the sterilized larvae. It seems that the symbionts of *Lasioderma* supply more cholesterol than those of *Stegobium*, also that the absence of sterol from the diet is more critical for *Stegobium* than *Lasioderma*.

Discussion

The results of the present investigation restate and largely confirm the conclusions drawn in an earlier investigation (Blewett and Fraenkel, 1944), that the intracellular symbionts of *Lasioderma* and *Stegobium* have a nutritional effect similar to that of yeast in a synthetic diet, namely to supply vitamins of the B-complex. They go beyond the earlier results in that folic acid and biotin have been added to the list of individual vitamins tested. Folic acid, like many of the other vitamins, does not seem to be necessary in the diet when the larvae have their ordinary complement of symbionts, but becomes a critical requirement in the absence of the symbionts. With biotin the results were not quite conclusive. Neither *Lasioderma* nor *Stegobium* larvae grow well in the absence of biotin, even in the presence of symbionts, while without symbionts, the *Lasioderma* larvae gave about the same growth pattern and the *Stegobium* larvae entirely failed to grow. There were some minor discrepancies between the present and earlier investigation which might be due to a variety of factors, such as differences in the basic diet (addition of biotin and folic acid), the casein, and the environmental factors, or the insect stock. In the earlier investigation, but not in the present one, choline was a critical requirement for the normal *Lasioderma* larva. On the former occasion choline chloride was a critical requirement for the sterilized *Stegobium* larva, while now the presence or absence of choline had little effect on both the normal and sterilized larva.

Results on the vitamin requirements of the *Stegobium* larva, which agree well with those given earlier by Blewett and Fraenkel (1944) and again in this publication, were recently reported by Lemonde and Bernard (1953), who succeeded in growing them on a diet which contained only two B-vitamins, thiamin and biotin. The larvae grew noticeably slower than on wheat flour; however, no improvement could be achieved by the addition of riboflavin, nicotinic acid, pantothenic acid, choline chloride or folic acid.

In general, the impression has been obtained that the symbiotic yeasts of *Lasioderma* exhibit a greater efficiency of action than those of *Stegobium*. *Lasioderma* yeasts in the *Stegobium* host have about the same effect as, and with regard to thiamin, an even better effect than, the *Stegobium* yeasts. *Stegobium* yeasts are less efficient in *Lasioderma* than the normal symbiont. This could be, however, attributed to the same unexplained phenomenon that *Lasioderma* yeasts are easier to culture than *Stegobium* yeasts. All these phenomena can be reduced to one common factor, namely the greater synthetic faculties of *Lasioderma* yeast. It would be interesting to compare vitamin contents of the two species of yeast, and also with those of known varieties of bakers or brewers yeast.

Finally the question remains to be discussed as to how the intracellular symbionts exert their nutritional effect. It has been reported earlier that toward the end of larval life, in the prepupa, the mycetoms gradually are reduced in size. Breitsprecher (1928) described that during this period large masses of yeasts, even whole mycetocytes, enter the lumen of the intestine and are eliminated with the faeces without digestion taking place. In our experience, yeast cells are liberated from the mycetoms all through the growing period of the larva, and only relatively few of these cells are found in the faeces (Fig. 2). It is therefore possible that the host acquires vitamins by digesting the symbionts. The alternative, difficult to prove, would be a diffusion of vitamins from the symbionts into the cytoplasm of the mycetocytes and hence to the hemolymph of the host. This process would differ little from the well known phenomenon of an enrichment of any microbial culture medium by the synthesis in, and diffusion from, the cultured microbe.

Acknowledgment

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Summary

1. The shape and appearance of the yeast-like symbionts of *Lasioderma serricorne* F. and *Stegobium paniceum* L. have been described. The symbionts have been cultured in Hansen's solutions and on an agar medium.

2. The larvae of both species are relatively little affected by the absence of certain vitamins of the B-complex. After elimination of the symbionts these vitamins become absolutely necessary.

3. Sterilized larvae of either species have been successfully re-infected with the symbionts of the other host, which then retain their peculiar shape and mode of action.

4. Cultured symbionts exert the same growth promoting effect as dried brewers yeast when added to the food.

5. The symbionts are also sources of sterols for their hosts.

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THE MOLTING CYCLE OF THE SPINY LOBSTER, PANULIRUS ARGUS LATREILLE. I. MOLTING AND GROWTH IN LABORATORY-MAINTAINED INDIVIDUALS[1]

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Since the summer of 1949, the author has become deeply interested in the physiology of molting and growth in the Bermuda spiny lobster, *Panulirus argus* Latreille. Investigations have been concerned with normal morphological and physiological changes related to molting and growth in this animal. Interest has centered around the changes which occur in the skeleton, tissues, blood, and urine of normal animals during the molting cycle.

The present paper, the first of a series, is concerned with the spectacular process of molting, the mechanics by which it is achieved, some physical factors influencing molting and growth, and growth determined by weight and length in laboratory-maintained spiny lobsters.

MATERIALS AND METHODS

Male and female spiny lobsters, ranging in carapace length from 20–92 mm., were generously supplied by the Bermuda Biological Station and Dr. W. H. Sutcliffe, who furnished the author with all immature forms from his catches of 1952. Only twenty-seven of approximately 500 animals supplied were of the carapace length range of 90–92 mm. These animals were included in the data for Figure 11. No further use was made of these animals following the first laboratory molt because Sutcliffe (1952, 1953) has pointed out that sexual maturity in females is attained some time after the carapace length reaches 90 mm. Information was almost exclusively limited, therefore, to animals of 20–89 mm. carapace length. All animals, when brought into the laboratory, were numbered with a lead or plastic tag, wired to the base of an antenna. Their number, sex, weight and carapace length were recorded. The animals were then placed in concrete tanks of running sea water (22′ × 3′ × 1½′). By partitioning portions of the sea water tanks with plastic screens, premolt animals could be isolated from the others. At the time of ecdysis animals were placed in individual aquaria or in partitioned portions of the tanks. Following ecdysis, animals were allowed to harden before being placed in partitioned

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[3] Contribution No. 214.

portions of tanks in different size groups. The laboratory history of each animal was followed closely throughout the period of observation by the method described. The method of designating stages in the molting cycle by letters A, B, C, D (Drach, 1939), as well as the method by which the molting cycle is divided into stages by daily intervals, was used.

The weights of animals throughout the molting cycle were obtained by carefully placing them on paper towels and drying them as thoroughly as possible. The animals were then rolled in a piece of nylon screen which restricted their movements while being weighed on a triple beam balance, accurate to 0.3 gm.

Length measurements were made with a vernier caliper from the mid-dorsal anterior point between the two rostral spines to the most posterior mid-dorsal point of the carapace.

In all cases where the standard error of the arithmetical mean was calculated, the method of Fisher (1948) for small samples was used.

With few exceptions, all animals were fed every other day. Their laboratory diet consisted of fish fry, anchovies, small pieces of larger fish, molluscs, and ground beef when the bait supply became low during the winter months.

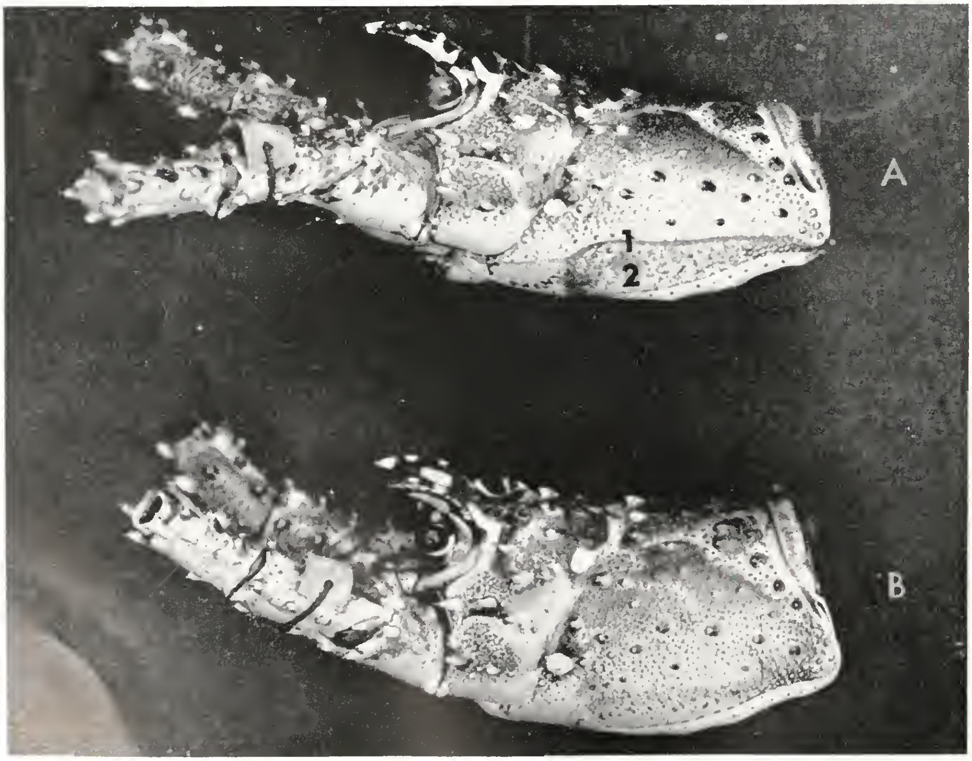


FIGURE 1. The shed (A) and hard carapace (B) of *Panulirus argus*. In the exuvium, the resorption or ecdysal line (1) and the area ventral to this, in which somewhat less resorption occurs (2), will be noted. Neither the ecdysial line nor the softened area ventral to the line will be noted in the hard carapace, which was removed from an animal in Stage C of the molting cycle.

Observations and Discussion

In *Panulirus argus,* a premolt animal can easily be detected by the appearance of a resorptive or ecdysial line along the branchiostegites (Fig. 1). This line becomes clearly evident 3–4 days preceding ecdysis in the summer months. Beneath this resorptive line appreciable softening (20% resorption—Travis, 1951a) of the whole ventral area occurs. Premolt animals can thus be detected by feeling this softened area even before the resorptive or ecdysial line is clearly evident. In the winter months, premolt animals can be detected by touch as early as 14–21 days

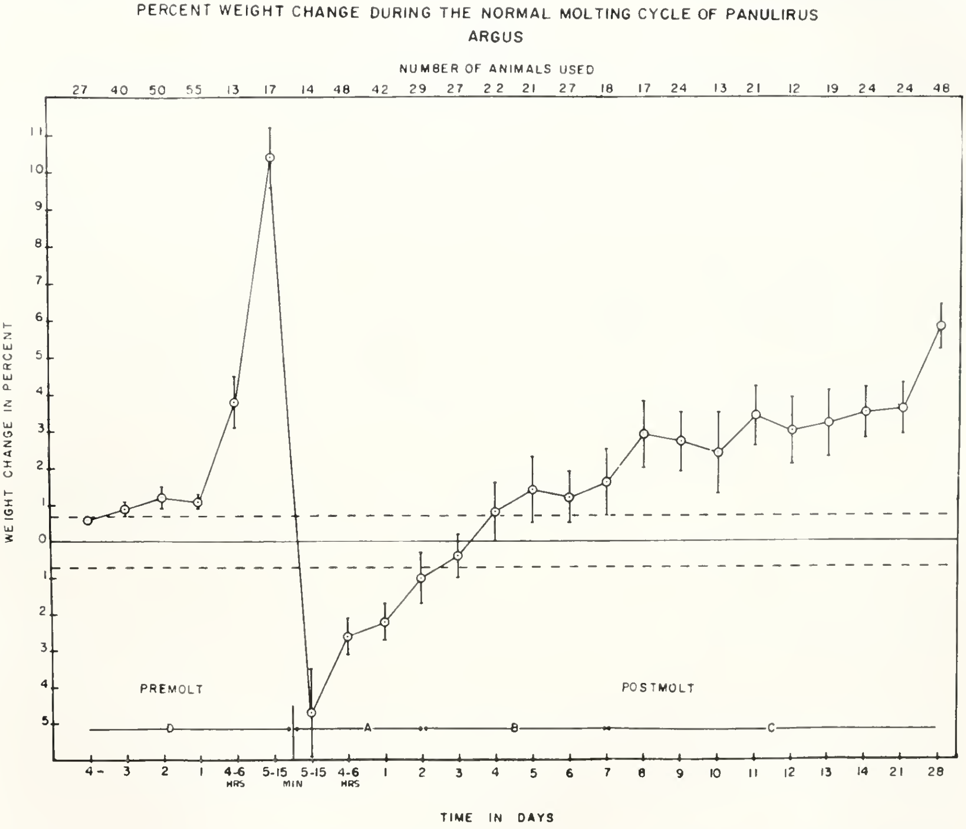


Figure 2A. The per cent weight change during the normal molting cycle of *Panulirus argus.* The number of animals used per point on the graph is indicated along the top ordinate. All points represent the mean arithmetical values obtained from the number of animals specified. The vertical lines represent plus or minus the standard error of the mean. The solid horizontal line marked by (o) on the graph, represents the base weight (intermolt weight) upon which per cent changes were calculated. Dashes above and below the solid horizontal line represent the standard daily variation in weight of 24 animals. This daily variation was observed over a 7-day interval during the intermolt period, a time at which the weight of the animals is stable. The vertical line at the bottom of the graph separates the premolt and postmolt period. The bottom ordinate represents the time in days preceding and following molting. The solid horizontal lines parallel to the bottom ordinate and broken by the letters D, A, B, and C designate the four major stages in the molting cycle as utilized by Drach, 1939.

preceding ecdysis. The lengthening of the premolt period is brought about by lower winter temperatures, which markedly slow down physiological activities associated with the molting process.

One of the most manifest external changes noted a few hours before ecdysis in *Panulirus* is the swollen condition of the intersegmental membranes separating the cephalothorax and abdomen. Due to resorption from the resorptive or ecdysial line, along the branchiostegites, and resorption from an articulating condyle connecting the branchial chamber to the posterior edge of the branchiostegites, lateral expansion of the soft body beneath the old skeleton is allowed. Such swelling and lateral expansion of the thorax immediately preceding ecdysis were first thought to be entirely due to the absorption of water. This has not proved to be the case and will be discussed in a subsequent section of this paper. In a search for the causal mechanisms immediately involved in molting, a number of factors were considered. These were weight changes, water uptake, and possible structures involved in water retention.

TABLE I

The per cent water absorbed and per cent weight increases preceding molting. Values are based on normal changes in weights associated with ecdysis in Panulirus argus. The abbreviations of Guyselman (1953) are used in this table

Number of animals used	Weight in grams						E as % of W_1	W_3 as % of I	W_1 as % of I
	I	W_1	E	E_1	W_2	W_3			
3	519.2–539.4	571.7–596.4	92.7–124.0	50.5–55.8	511.2–544.0	40.3–70.0	15.6–21.7	7.5–13.5	10.1–11.6
Avg.	528.5	584.4	111.9	53.0	524.1	51.9	19.2	9.8	10.9
3	623.3–668.9	663.5–730.1	103.3–140.5	56.0–65.2	620.0–663.5	30.8–73.9	15.6–19.2	4.7–11.0	6.5–9.3
Avg.	647.9	704.2	124.5	60.6	636.5	56.9	17.6	8.8	8.3

I = Wet weights of intermolt animals
W_1 = Wet weights of animals 15 minutes preceding molt
E = Wet weights of exuviae
E_1 = Dry weights of exuviae
W_2 = Wet weights of animals 15 minutes following molt
$W_3 = W_2 - (W_1 - E)$ = Weight of water absorbed in a period taken between 15 minutes preceding to 15 minutes following molt
E as % of W_1 = The weight of the exuviae, expressed as per cent of the total premolt wet weight of the animals
W_3 as % of I = The weight of the water absorbed expressed as percentage of the previous intermolt wet weight
W_1 as % of I = Total premolt weight increase expressed as per cent of the previous intermolt wet weight

Weight changes

In *Panulirus,* marked changes in body weight are observed immediately preceding ecdysis and for a four-week period following it. These wet weight changes, expressed as per cent of the previous intermolt weights, were followed throughout the normal molting cycle of the spiny lobster and are shown in Figure 2A. It will be observed in the 80–89 mm. carapace length animals, represented in this graph, that little change in weight is noted in the pre-ecdysial period until the day preceding ecdysis. At this time, the first significant weight increase is observed a

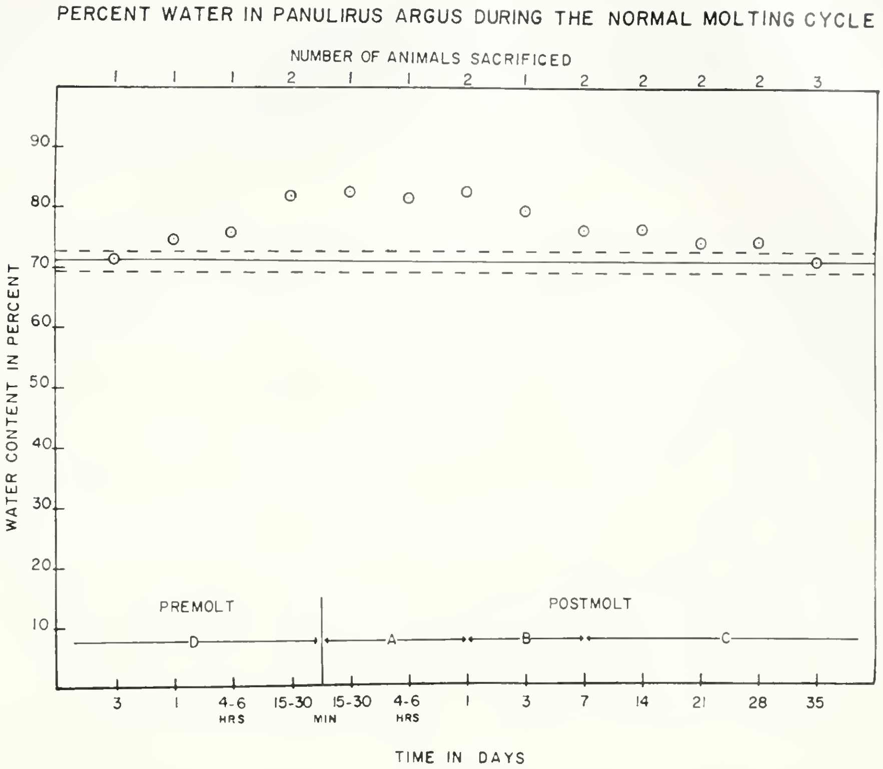


FIGURE 2B. Changes in water content of *Panulirus argus* during the normal molting cycle. Dashes above and below the horizontal line indicate plus or minus variations from the intermolt mean.

few hours preceding molting. The weight of the animals continues to increase to a peak premolt value of around 10%. This peak premolt value is attained 5 to 15 minutes preceding ecdysis. The marked increase in weight attained at this time is primarily the result of water absorbed.

During the early post-ecdysial period (15 minutes) a striking weight loss is observed. This weight loss of almost 16% is primarily due to the loss of the old exuviae, which constitute from 15.6 to 21.7% of the total premolt wet weight of the animal (Table I). Within 4 to 6 hours, however, the weight begins to rise. This continues at a rather steady pace, reaches the intermolt value two to four days following molt, significantly rises above this level at eight days, tends to level off and reaches the new intermolt value between 28 and 35 days.

Water uptake

It was mentioned earlier that the peak premolt weight increase of 10% was the result primarily of water absorbed. This is indicated in Table I. Here the weight of the water absorbed preceding molt is determined (Drach, 1939; Guyselman, 1953) and expressed as per cent of the previous intermolt wet weight. The premolt weight increase, expressed as per cent of the previous intermolt wet weight determined as indicated in the previous section, is also listed. The weight of water absorbed and the total premolt weight increase, both obtained by two different procedures (Table I), compare favorably. Weight increases preceding molting, therefore, are entirely due to water absorbed. This is evident both in animals within the 500 gram range and those in the 600 gram range.

Water content

The water content of the animals, expressed as per cent, was determined from the wet and dry weights of individuals sacrificed at various intervals throughout the molting cycle. It will be observed (Fig. 2B) that the water content of an intermolt animal (late stage C) is 71.0%. The water content of *Panulirus* begins to increase the day preceding molt (74.7%), rises to 81.3% fifteen minutes preceding molt, and reaches a peak value of 82.4% one day following molt, 11.4% above the intermolt value of 71.0%. It should be pointed out here that the increase in water content of individuals sacrificed 15–30 minutes preceding molt (Fig. 2B) is in agreement with the weight increase observed in Figure 2A and the calculated per cent water absorbed in this same period (Table I). Since presumably all mineral and organic resorption is completed in the old skeleton at 15–30 minutes preceding ecdysis, the wet and dry weights of these skeletons must be subtracted from the total wet and dry weights of the animals with their old skeletons, to obtain the true water content of the animals alone. If this is not done the weight increase of 10% is observed (Fig. 2A) with no rise of corresponding magnitude in water content, which is represented in Figure 2B. Because all animals used in Figure 2B were sacrificed for dry weight determinations, the average per cent of the total weights of the animals due to exuviae, both wet and dry weights, was calculated from values given in Table I. These values were then applied to the two animals sacrificed 15–30 minutes preceding molt (Fig. 2B) to obtain the presumed wet and dry weights of the old skeleton of these animals. The water content of these animals, without their old skeletons, was then calculated. From three days following molt the water content slowly declines, but does not return to its intermolt range of 71.0% before 21 to 28 days. It might be mentioned that at 28 days the animals have fully calcified their new skeletons (the principal layer and the membranous layer are fully formed), weight stability is reached, and apparently all growth of soft tissues is completed. It would appear from Figure 2B that water absorption is not completed for at least two weeks following molting. This is perhaps a false impression one might obtain at first observation. Although the water content is above the late Stage C value, it must be remembered that growing tissue is gradually replacing water absorbed at and following molt. Water absorption in *Panulirus* is probably completed the day following molt. This is in agreement with the situation observed in *Maia squinado*, *Carcinides maenas*, and *Cancer pagurus* (Drach, 1939) in which water absorption apparently is completed within 6 to 12 hours or less following molt.

Mechanisms Involved in Swelling and Lateral Expansion of the Thorax Immediately Preceding Ecdysis

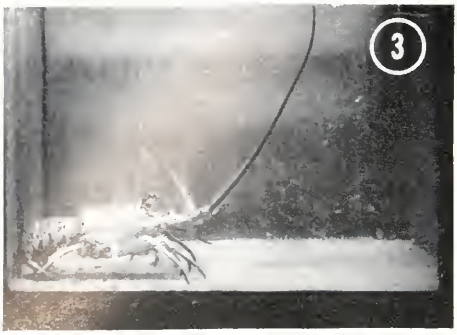
Since the animals increase their body weight approximately 10% before molting and since this increase is entirely accounted for by uptake of water, the question arises as to how this water enters the organism. Two possible structures which might be involved in water retention were studied with the idea of obtaining evidence of the cause of the greatly expanded and swollen thorax, which results ultimately in the lifting of the exuviae at ecdysis. The major structures considered were the membranous layer of the old skeleton and the stomach.

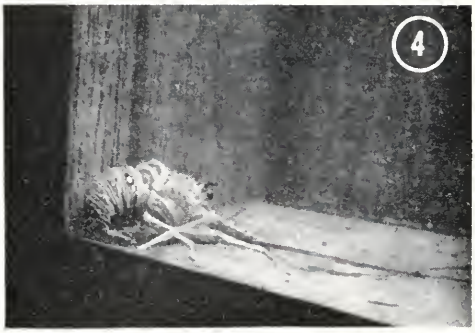
The membranous layer

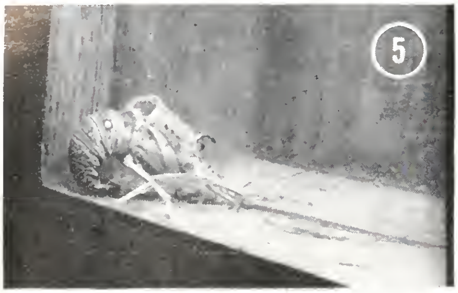
The homogenous non-laminated layer which is attached to the inside of the exuviae, observed by Vitzou (1882), Herrick (1895), Yonge (1932), and called the membranous layer by Drach (1939), was investigated as a possible site of water retention. It might be added that this layer holds large amounts of water after the exoskeleton is shed and appears to swell because of water absorption from the medium. Weight changes were followed in two premolt animals. From one animal (A) the membranous layer was removed at one day preceding molt, at which time no marked change in body weight had occurred, while in the other (B) the membranous layer was removed when the animal had increased its body weight 8% preceding molt. Wet and dry weights of pieces of this layer (as close to 2 cm^2. in size as possible) were obtained and per cent water in each piece compared. A difference in water content of the two pieces might indicate whether this structure is involved in water retention. It was found, however, that the water content in (A) was not appreciably different from that of (B). Therefore the idea that water retention by the membranous layer may contribute to weight changes preceding molt was abandoned.

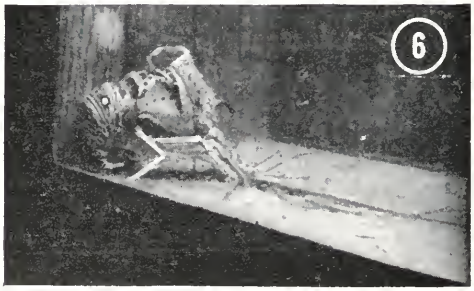
The stomach

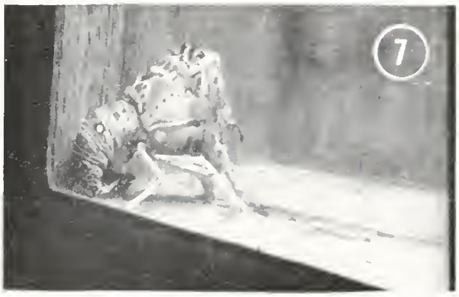
The stomach was then considered as a site for water uptake and retention preceding molt. Drach (1939) demonstrated in crabs that absorption of water during the course of shedding and shortly following occurs primarily through the gut. The stomach becomes turgid and greatly swollen during this period and in *Panulirus* swells to the extent that it occupies over half of the cephalothorax. This would cause pressure to be exerted on the surrounding regions and is, in all probability, the actual cause of swelling and lateral expansion of the thorax. Drach (1939) attributed the turgidity and swelling of the stomach in *Maia squinado* and *Cancer pagurus* to the water held in the stomach and digestive tube during shedding. He was able to remove a considerable quantity of water from the stomach of these animals, shortly preceding ecdysis, and within a short interval removal of a large quantity was again possible. In an attempt to withdraw water from the stomach of *Panulirus,* 10 premolting animals were selected. As the animals increased their weights preceding ecdysis, attempted withdrawal of water from the stomach, by inserting a cannula into the stomach via the esophagus, proved fruitless. Even when the increase in body weight had reached 8% a few minutes preceding ecdysis, little water could be withdrawn. No more than 0.5 cc. could ever be withdrawn. In three cases, animals were weighed until the body weight increased by 8–10% and then sacrificed. The old and new skeleton overlying the stomach were removed. A syringe needle was inserted in the turgid stomach in an attempt to withdraw fluid. No fluid was present. On the contrary, it was

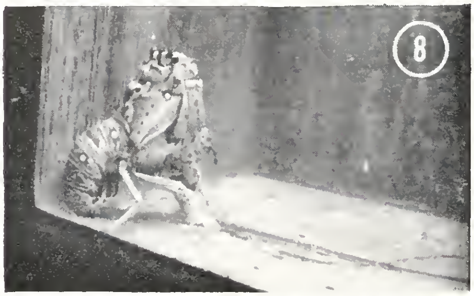


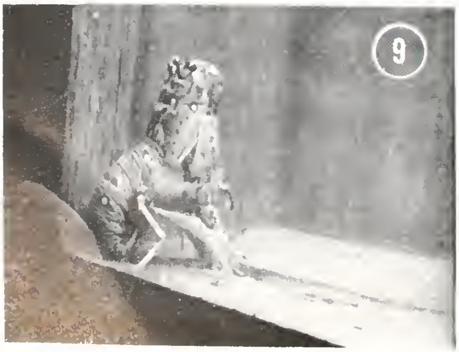












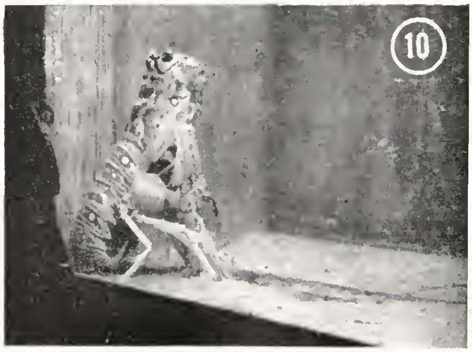


FIGURE 3. Ecdysis in *Panulirus*. The animal seeks out a suitable place to shed.

FIGURE 4. The tail is flexed; the thorax is lowered on the substratum with antennae and antennules lowered and extended to facilitate forward bracing of the animal, while the last pair

found that the turgidity and swollen condition was due to the presence of gas of unknown composition which appeared to be between the old stomach lining and the new. Following the puncture of the new outer layer with a syringe needle the wound closed and the stomach again swelled. An incision in this layer caused the stomach to collapse. The "balloon" condition of the new stomach causes the new soft body to expand in the thoracic region and is without doubt the immediate mechanism which lifts the old carapace at ecdysis. Such a mechanism would also account for the increase in volume of the emerging animal.

Water absorbed preceding ecdysis is probably taken in primarily through the gills and the soft intersegmental membranes. It does not appear to be taken in through the digestive tract. Hydration of the tissues probably occurs during the late pre-ecdysial as well as post-ecdysial period. This hydration of the tissues has been indicated by Baumberger and Olmstead (1928) in *Pachygrapsus crassipes* and is suggested by the recent work of Kincaid and Scheer (1952) on *Hemigrapsus nudus*. It is pertinent to add that the former workers showed that there was a rise in the internal osmotic pressure preceding molt. This peak in osmotic pressure is presumably caused by an increase in osmotically active constituents (organic acids) which accumulate in the hemolymph as a result of the breakdown of fats and glycogen, abundant in the hepatopancreas during the premolting period of all Crustacea investigated. The increase in osmotic pressure preceding molt would therefore account for increased water absorption preceding and immediately following molt. Increased water uptake causes the osmotic pressure to fall sharply following molt and to return gradually to normal as the skeleton of the animal hardens. A study of the green glands during the pre-ecdysial period should be made. It is quite possible that antidiuresis might occur during the few hours preceding ecdysis. Such water retention could also serve as one of the correlatives contributing to the marked increase in weight of the animal during this time. Furthermore, it is

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of walking legs is extended behind and serves as the hind bracing elements. The old carapace has begun to lift, making visible the new skeleton below the old branchiostegites.

FIGURE 5. A further stage during ecdysis showing the progressive lifting of the old carapace.

FIGURE 6. At this stage the animal has raised the thorax off of the substratum and the old carapace has lifted about 45° from its original position.

FIGURE 7. At this stage of ecdysis the animal has raised itself considerably from the substratum with the tail gently flexed. The old carapace has lifted and has tilted at an angle of 90° from its original position. The thorax of the animal is almost completely freed, making visible the new eyes and rostral spines. During this stage of ecdysis, the old branchial chamber becomes evident ventral to the new branchiostegites. It is interesting to note that the abdomen begins to undergo contractions in a telescopic fashion, *i.e.*, segments begin to telescope and extend. Freeing of the abdomen occurs in this manner.

FIGURE 8. A further stage in ecdysis. The animal has raised itself considerably from the substratum by arching its soft cephalothorax. The old branchial chamber is almost completely freed. The new antennae are just beginning to appear and two new abdominal segments are free.

FIGURE 9. In this stage of ecdysis, the animal has arched its soft cephalothorax almost 90° from its original position, three new abdominal segments are freed, the fourth beginning to appear and the new antennae are much more evident. The new 5th walking leg may be observed between the old gill chamber, old branchiostegites and the new.

FIGURE 10. The last stage of ecdysis, a second or so before the animal gives one sudden flexion and extension of the abdomen freeing itself completely from the old skeleton. It will be noted that four new segments are visible, the new proximal portions of the antennae and the new fifth walking leg are clearly evident.

possible that changes in salt excretion by the green glands might also be a factor in raising the osmotic pressure prior to molting.

Ecdysis

All of the changes observed in the premolt period lead to shedding of the exoskeleton. This is depicted in Figures 3–10. For a period of at least two hours preceding ecdysis, the spiny lobster undergoes exercise movements. These include flexing of the abdomen under the thorax and bumping it along the substratum; lowering of the thorax on the substratum while raising and extending the abdomen rigidly; lunging movements with sudden stops. These are all movements to facilitate loosening of the old skeleton from the new. Following such movements, the animal seeks a suitable place to shed (Fig. 3). The tail is then flexed; the thorax is lowered on the substratum with antennae and antennules lowered and extended to facilitate forward bracing of the animal, while the last pair of walking legs is extended behind and serves as the hind bracing elements (Fig. 4). In this position, little movement is observed other than slow contractions of the soft body beneath the old carapace. As these slow contractions occur the old carapace begins to lift at the intersegmental membrane between carapace and abdomen, making visible the new skeleton below the old branchiostegites (Figs. 4, 5). It should be pointed out again that the resorptive or ecdysial line serves as a hinge. The resorption from, and loosening of, an articulating condyle, which attaches the branchial chamber to the inner posterior edge of the branchiostegites, allow the soft body to expand in width (Figs. 5, 10). There is no breaking along the resorptive line of *Panulirus argus* as Crawford and De Smidt (1922) and Smith and Gatham (1948) thought. As the eyes are withdrawn from their old skeleton, the carapace lifts considerably (Fig. 7). At this time (Figs. 7, 10) the segments of the abdomen begin to move in and out in a telescopic fashion, thus freeing themselves from the old skeleton. As the animal pulls itself free from the old carapace it raises itself from the substratum by arching its soft cephalothorax (Figs. 8, 10). The rostral spines and eyes appear, while the old gill chamber can be seen ventral to the new branchiostegites (Figs. 7, 10). The antennae are then pulled free of the old skeleton (Figs. 8, 10). Newly withdrawn legs may be observed between the gill chamber and branchiostegites of the old skeleton (Figs. 9, 10). Three abdominal segments are observed free of the old (Fig. 9). A later stage (Fig. 10) indicates how the animal frees itself from the old skeleton. Here four abdominal segments are freed and the legs are more clearly observed above the old gill chamber between the new and old branchiostegites. The animal raises itself much above the old skeleton while pulling itself free. At this stage the animal suddenly flexes and then extends the abdomen, freeing it completely from the old skeleton. The actual time required for the entire process of ecdysis averages from 3–5 minutes for the 70–79 mm. animals depicted. The larger animals, 80–89 mm., require from 5–10 minutes. Immediately following ecdysis the animal moves about vigorously. Expansion of the flattened appendages is accomplished in this manner. By such movements, blood rushes into the appendages and causes them to assume their normal shape.

Following molting, these animals were allowed to remain isolated from all others through Stage A and B (a period of 5–6 days) at which time they begin to feed, their skeleton attains a parchment-like consistency, and they are capable of protecting themselves. By 7–12 days (Stage C) the branchiostegites have hardened and complete hardening of the skeleton by deposition of minerals therein occurs within approximately four weeks.

Factors Influencing Molting

Temperature

In Bermuda where average monthly water temperatures range from approximately 17° in February to 29° C. in August, the range would appear to be sufficiently great to stimulate *Panulirus argus* to molt seasonally. Very little molting occurs in the months of December–April, during which time the water temperatures are lowest (Fig. 11 and Table II). It might be added that the temperature of tank water is within a few tenths of a degree of that recorded at the nearby tide station. A water temperature of 22° C. or slightly over in the spring is sufficient to initiate molting (Fig. 11). As water temperatures approach 23° C. the number of molting individuals rises from below 10% to well above. This rise begins in May and increases throughout the summer months with the peak of molts occurring in June, July, August and September, at which time the highest water temperatures of 28°–30° C. are reached. The number of molting individuals, during

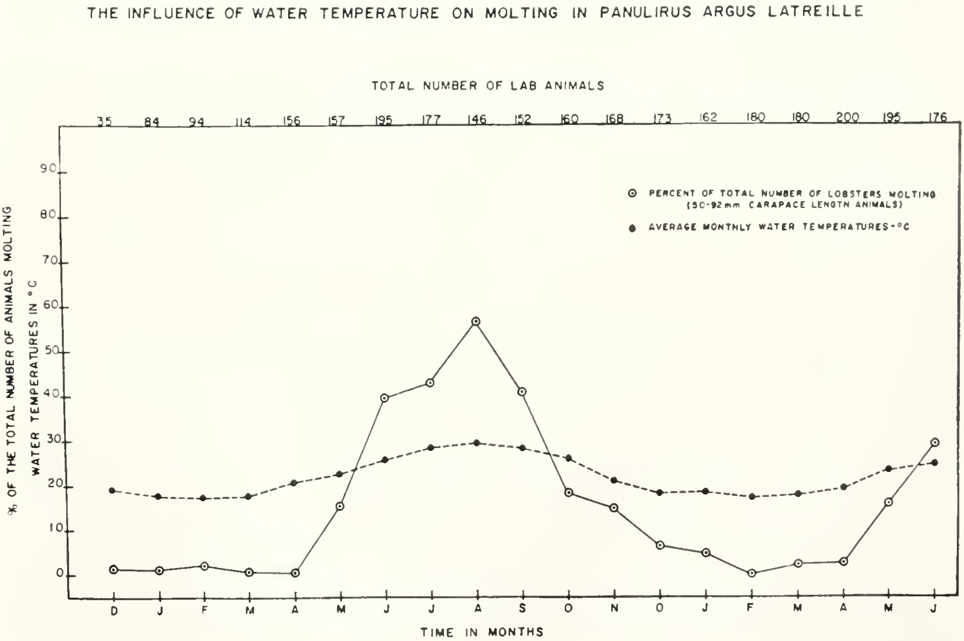

FIGURE 11. The influence of water temperature on molting in *Panulirus argus*. The total number of animals in the laboratory population in any given month is indicated along the top ordinate while the respective months are indicated along the bottom ordinate. Along the Y-ordinate water temperature in ° C. and per cent of the total number of animals molting are indicated. The per cent of molting individuals is plotted with mean temperatures against time in months.

late fall, begins to decline as temperatures decrease, and in the month of December (temperature range 18°–20°) less than 10% of the total laboratory population molts. Water temperatures of 17°–18° C. virtually stop the process. The influence of the water temperatures on molting has been pointed out by Templeman (1936a, 1936b, 1940), Drach (1939, 1949), and Truitt (1939). The quantity of food required by the animals also depends largely upon the temperature. When the water temperature is low the food requirement of the animals is low. Their metabolism is greatly accelerated with a rise in water temperature. Other factors, such as photoperiodism, lunar phases, tides, and salinity of the water which might possibly influence molting, have not been investigated by the author. Templeman (1936b), however, in addition to showing that at high water temperatures larval American lobsters molted more frequently than in colder waters, found that salinities as low as 21 parts per mille were only slightly less favorable than the normal 31 parts per mille. Larvae reared in total darkness molted earlier than those reared in light, were larger, and had a higher survival rate than those reared in light. He also found (1940) that egg-laying in American lobsters was retarded by cold.

Table II

Intermolt periods of 80–89 mm. carapace length animals during a period of one year

Month of molt	Number of animals molting	Intermolt periods in days	Average monthly Temperature, °C.
January	3	161±1	17.9
February	0	No molts	17.4
March	0	No molts	17.8
April	1	73	20.7
May	10	67±2	22.8
June	17	68±2	25.9
July	13	60±4	28.4
August	11	142±8	29.2
September	10	201±6	28.1
October	5	232±5	26.0
November	5	213±2	21.0
December	0	No molts	18.2

It will be noted from Table II that the intermolt or growth periods are the shortest in animals molting in the months of May, June, and July. These animals, after molting in the respective months, are subjected to gradually rising temperatures and molt again within 60–70 days. Although the highest average monthly temperature occurs in August, animals molting in this month will be subjected to gradually falling temperatures in the following months. As a consequence, their metabolism is slowed down, feeding becomes less, and their intermolt periods are prolonged. This is likewise true of animals molting in September, October, November, and January. The growing period of the Bermuda lobster therefore appears to be limited effectively to a period of approximately seven months in the laboratory (the months during which the water temperatures are highest). Very little molting and growth occur in the population during the months of December through April (the months during which the water temperatures are lowest). Sutcliffe (personal communication) has indicated that the two foregoing statements are also, in general, applicable to the field population.

Although the summer intermolt periods (Table III) are shorter in smaller than in larger animals, the actual number of molts observed during a period of one year in four of the size groups maintained in the laboratory proved to be approximately the same. Generally four molts per year were observed in these groups. This again appears to be due to the fact that the intermolt periods are prolonged following the August molt. As winter months approach, the smaller as well as the larger sexually immature animals cease to molt and grow.

The influence of size on molting

From the foregoing, water temperature does appear to be largely responsible in *Panulirus argus,* for: (1) The onset of molting; and (2) the frequency of molting and therefore the rate of growth. It is apparent that the rate of growth varies from one size group to the next. The smaller the individuals, the more frequently they molt or the more rapid are their growth rates. This was determined by noting the summer intermolt periods of each size group maintained in the laboratory (Table III). It is evident from Table III that as animals increase in size their intermolt periods are longer or the frequency of shedding decreases. In small animals, the rate of growth is rapid and it decreases progressively as they approach sexual maturity. Kinoshita (1934), Nakamura (1940), Templeman (1940), Challenger (1943), Pearson and Anderson (1946), and Bradstock (1950) have also noted that young lobsters of several species (*Panulirus japonicus, Homarus americanus, Panulirus interruptis,* and *Jasus lalandi*) molt more frequently than old ones.

TABLE III

Average summer intermolt periods of various size lobsters molting in May, June and July

Number of animals	Carapace length	Average summer intermolt period ± Standard Error of the Mean	Theoretical number of molts per year in the laboratory based on yearly intermolt periods and growing time	Actual number of molts observed in animals maintained for one year
41	80–89	65±1	3–4	3–4
42	70–79	56±1	3–4	3–5
25	60–69	55±2	3–4	3–4
5	50–59	51±2	4–5	4
4	40–49	41±1	5	Not carried for one year
11	30–39	44±3	5	Not carried for one year
29	20–29	40±2	5	Not carried for one year

GROWTH IN LABORATORY-MAINTAINED ANIMALS

Before discussing growth of laboratory-maintained spiny lobsters, it should be pointed out that this information cannot represent exactly the growth of animals under natural conditions, for laboratory conditions, at best, do not truly duplicate the field situation.

The following information was obtained to serve as a basis for future studies concerning hormonal factors which influence molting and growth in the Bermuda spiny lobster.

Growth by weight

Weight stability is reached several weeks following a molt. The length of this period depends upon size of the animals and the season of the year. No attempt was made in these sexually immature animals to separate the data on the basis of sex. Growth in weight is one of the most important means of studying many aspects of the molting cycle, such as determining the per cent water in the animals, the amount of water absorbed, and per cent weight change throughout the molting cycle (discussed in a previous section of this paper.) Special care, however, must be taken in handling Crustacea while following individuals through their cycle, because the antennae and periopods may be autotomized. If such happens, these animals can no longer be used for subsequent weight changes. Autotomy can generally be avoided by draining the large concrete tanks and by carefully lifting each individual out of the drained tanks for weighings.

TABLE IV

Per cent growth by weight per molt during the summer months

Number of animals	Carapace length (mm.)	Mean per cent growth by weight per molt ±standard error of the mean	Calculated per cent growth per year based on number of molts per year (Tables III, VI)	Weight stability is reached in the summer days post molt
48	80–89	5.8±0.6	17–23	28–35
33	70–79	8.4±1.0	25–34	28
22	60–69	10.0±1.1	30–40	28
20	50–59	14.9±1.7	60–75	21–28

The weight changes of the 80–89 mm. carapace length animals were observed throughout the molting cycle (Fig. 2A). These weight changes are expressed as per cents of the previous intermolt weights. The per cent growth by weight of these larger animals, throughout the summer months, indicates that they increase their body weight on the average approximately 6% per molt in the laboratory. From Table IV it will be observed that the smaller the animals, the greater the per cent growth by weight. Templeman (1940) has previously shown that the absolute increase in weight per molt of the American lobster is greater with an increase in size. An American lobster 6″ weighing 3.5 oz. gains 2 oz. per molt, (5.7%), while a 14.5″ lobster weighing 55 oz. gains 22 oz. per molt, (4.0%). Weight stability in *Panulirus* is reached earlier in smaller animals than in large ones. The period at which the skeleton is fully hardened and at which time growth of the tissues is virtually completed (Late Stage C of Drach, 1939) must be utilized in determining the per cent growth by weight. Earlier stages in the molting cycle, A, B, or early C, will give fluctuating results simply because there is a rather steady increase in weight up to Late Stage C (28 days) in *Panulirus*.

Growth by length

Growth in length is determined by the growth of the epidermis, which retracts from the old skeleton and increases its cell number or size or both about ten to fourteen days before shedding occurs in the summer months. It is at this time of retraction that the epidermis grows before the new soft skeleton begins to be deposited. Increase in length may be noted within an hour following ecdysis. By this time, the new soft skeleton has expanded to the point at which *Panulirus* reaches a size which it will retain until the following molt.

The per cent growth in length per molt for the various size classes is listed in Table V—a. It might be pointed out at this time that Tables V—a and V—b represent two different groups of animals.

It will be noted that there is a greater per cent growth by length per molt in smaller than in larger animals. This has also been observed in *Homarus americanus* by Templeman (1936a, 1940), Mackay (1926, 1929); in *Cancer magister* by Mackay and Weymouth (1935); in *Panulirus japonicus* by Kinoshita (1934) and Nakamura (1940). This difference observed in the laboratory does not appear to be due to significant differences in the gain of carapace length per molt from one size group to the next, but appears to be due to the fact that a smaller animal increasing its carapace length the same amount as a larger one (Table V—b) shows a greater per cent growth by length. There is a tendency, though not a significant one, for the smaller animals (40–59 mm. class) to increase their carapace length more per molt than the larger animals (Table V—b).

TABLE V

V—a Per cent Growth by Length per Molt				*V—b* Gain in Carapace Length per Molt	
Carapace length (mm.)	Number of animals	Mean per cent growth by length per molt ±Standard Error of the Mean	Calculated per cent growth per year based on number of molts per year (Tables III, VI)	Number of animals	Average (mm.) gain in carapace length per molt ±Standard Error of the Mean
80–89	72	2.5±0.5	8–10	106	2.6±0.2
70–79	73	3.2±0.3	10–16	131	2.8±0.1
60–69	53	4.4±0.4	13–18	102	3.1±0.2
50–59	21	5.8±0.7	23–29	45	3.2±0.2
40–49	15	8.9±0.8	45	27	3.6±0.5

A few animals of the first four size groups (50–89 mm. carapace length) were successfully maintained for one year in the laboratory (Table VI). The number of molts, total number of mm. gained, and the per cent increase in length per year were determined. The results observed here serve as a rather good cross-check on information obtained in Table V—a and V—b from other animals.

Molting without growth

That growth by length does not necessarily accompany molting, was evidenced in some laboratory-maintained animals. Molting without growth or negative growth has also been observed in *Panulirus argus* maintained in live cars or wire enclosures, under what this author considers as extremely poor conditions, by Marshall (1945) and Dawson and Idyll (1951). Within a period of almost two years and out of a total number of nearly 500 laboratory animals of various sizes, 27 were observed to undergo no increase in length following the first laboratory molt. Five of the 27 animals decreased in length by one mm. following molt. Seventeen of the 27 lobsters were obtained from a small, overcrowded lobster pound in which food was scarce and in which, as a result of overcrowding, most of the animals had autotomized some periopods as well as antennae. Autotomy, as a result of handling, was occasionally observed in animals brought in from the field, 10 of which are included in the 27 mentioned above. That loss of appendages markedly affects the growth of the animal as a whole was demonstrated in the American lobster by Emmel (1904, 1906). He found that when regeneration occurs, the regenerating appendages are favored over increase in body length at the first molt after injury, and that regeneration tends to lengthen the intermolt period. A previous history of unfavorable environmental conditions, such as insufficient food, overcrowding and autotomy of appendages, did not result in growth at the first molt in the laboratory, although subsequent molts by these animals were accompanied by good growth.

Table VI

Growth in length of animals maintained for one year in the laboratory

Class	Number of animals	Number of molts per year	Carapace length gain (mm.) per year	Mean per cent increase in length ± Standard Error of the Mean
80–89	13	3–4	9.1	10.7 ± 1.0
70–79	15	3–5	9.7	13.2 ± 0.9
60–69	13	3–4	12.2	19.1 ± 1.1
50–59	2	4	11.0	21.2 ± 2.0

Summary

1. In *Panulirus argus*, premolt animals may be detected by the appearance of a resorptive or ecdysial line along the branchiostegites.

2. One of the most obvious external changes in *Panulirus* a few hours before ecdysis is the swollen condition of the intersegmental membranes.

3. Five to fifteen minutes preceding ecdysis, animals of 80–89 mm. carapace length increase their body weight by approximately 10%. This weight increase begins four to six hours preceding ecdysis and is entirely due to water absorption. There is a loss of weight following ecdysis due to loss of the exuviae. Within a few hours following molt, the weight of the individual begins to rise and continues to do so until a maximum is reached at 28–35 days. The water content begins to rise the day preceding molt, reaches its highest peak (11% above the intermolt value of 71%) one day following molt, slowly declines but does not return to its intermolt value before 28–35 days. At this period the animal has fully calcified its new skeleton, and apparently all growth of soft tissues is completed. Weight stability is not attained in 80–89 mm. carapace length animals until 28–35 days following molt.

4. Due to resorption from the ecdysial line along the branchiostegites and resorption from an articulating condyle connecting the branchial chamber to the posterior edge of the branchiostegites, progressive swelling and lateral expansion of the thorax is allowed.

5. Turgidity within the new stomach causes considerable pressure to be exerted on the surrounding regions and results in the progressive swelling and lateral expansion of the thorax preceding ecdysis. This is due to a gas which appears between the old stomach lining and the new.

6. Photographs and captions depict ecdysis in *Panulirus*.

7. The molting process appears to be directly correlated with water temperature. In Bermuda average monthly water temperatures range from 17° in February to 29° C. in August. Little molting occurs in the months of December through April, during which time the water temperatures are lowest. Water temperatures of 22° C. in May are sufficient to initiate molting. The number of molting individuals in the laboratory population increases throughout the summer months with a maximum in June, July, August, and September, at which time the highest water temperatures of 28–30° C. are reached. This means that the intermolt periods are shortest during the summer months. The number of molting individuals during late fall declines, as temperatures decrease. Water temperatures of 19° C. or below, however, are required to stop the molting process in the winter. Decrease in molting frequency in the fall seems to be a consequence of decreased metabolism.

8. The growing period of the Bermuda lobster appears to be limited effectively to approximately seven months in the laboratory (May–November).

9. Smaller individuals grow more rapidly in the summer months than do larger individuals. Although the summer intermolt periods are shorter in smaller than in larger animals, the actual number of molts observed during a period of one year in four of the size groups maintained in the laboratory (50–89 mm. carapace length) proved to be approximately the same. An average of four molts per year was observed in these groups. As winter months approach, the smaller as well as the larger animals cease to molt and grow.

10. The smaller the animals, the greater the per cent growth by weight. Larger animals, however, gain more actual weight than smaller animals per molt.

11. There is a greater per cent growth by length per molt in smaller than in larger animals. The difference observed between larger and smaller animals is an apparent one because a smaller animal increasing its carapace length the same amount as a larger one shows a greater per cent growth by length.

12. Growth by length does not necessarily accompany molting. This was evidenced in some laboratory-maintained animals. In virtually all of these cases the environmental conditions of the individuals were poor.

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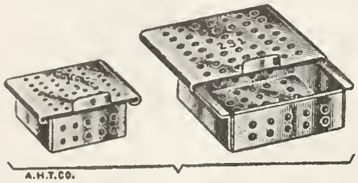

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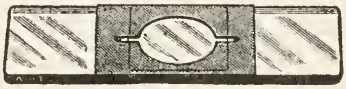

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